Phagocytosis of *Trypanosoma congolense* by macrophages:

The role of IgM antibody to variant surface glycoprotein (VSG)

A Thesis
Submitted to the College of Graduate Studies and Research
In Partial Fulfillment of the Requirements for the Degree of
Master of Science
In the Department of Veterinary Microbiology
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ABSTRACT

Trypanosoma congolense is a single-cell blood parasite and an important pathogen causing African trypanosomiasis, also called n'gana, in livestock. N'gana in cattle is a chronic disease associated with anemia, cachexia and increased susceptibility to secondary infections. Infection of mice can be used as an experimental model to study the host-parasite relationship. As determined by their survival time, BALB/c mice are highly susceptible to *T. congolense* infection, whereas C57BL/6 mice are relatively resistant. The surfaces of African trypanosomes are covered with a layer of a single species of glycoprotein, called variant surface glycoprotein (VSG). Production of antibodies to the VSG of African trypanosomes is one of the major immune responses leading to control of parasitemia. The reaction of antibodies with VSG of trypanosomes, for presently unknown reasons, predominantly activates the alternative complement pathway rather than the clasical pathway of complement. IgM antibodies are the first and predominant class of anti-trypanosomal antibodies in infected animals. Antibody-mediated phagocytosis of *T. congolense* by macrophages is considered a major mechanism of control of parasitemia, besides antibody/complement-mediated lysis and cytotoxic effect by macrophage-derived nitric oxide (NO). The receptor(s) on macrophages that recognizes IgM antibody-coated trypanosomes and enables their phagocytosis is unknown. Interaction of antibodies with the VSG of trypanosomes not only causes phagocytosis of trypanosomes by macrophages, but also leads to the release of sVSG from the trypanosomes. sVSG has been found to modulate various functions of

the host: induction of polyclonal B cell activation and modulation of macrophage functions, such as the induction of TNF-α synthesis and the inhibition of IFN-γ-induced nitric oxide production. The objectives of this thesis are:

1) to test whether CR3 (Mac-1; CD11b/18) is involved in IgM anti-VSG-mediated phagocytosis of *T. congolense* by macrophages,
2) to test the effects of anti-VSG antibody and complement on the release of soluble VSG from *T. congolense*

1) When the trypanosomes were incubated with IgM anti-VSG antibody and fresh mouse serum, fragments of complement component C3 were found to be deposited onto *Trypanosoma congolense*. Thus, it was assessed whether complement receptor CR3 (CD11b/CD18; receptor for iC3b) might be involved in IgM anti-VSG mediated phagocytosis of *T. congolense*. In the presence of fresh mouse serum, there was significantly and markedly less phagocytosis of IgM-opsonized *T. congolense* by CD11b-deficient macrophages compared to phagocytosis by normal macrophages (78% fewer *T. congolense* were ingested per macrophage). There also was significantly less TNF-α (38% less), but significantly more NO (63% more) secreted by CD11b-deficient macrophages that had engulfed trypanosomes than by equally treated normal macrophages. It was concluded that CR3 is the major, but not the only, receptor involved in IgM anti-VSG-mediated phagocytosis of *T. congolense* by macrophages. It was further concluded that signaling via CR3, associated with IgM anti-VSG-mediated phagocytosis of *T. congolense*, either directly or indirectly, enhances synthesis of disease-producing TNF-α and inhibits the synthesis of parasite-controlling NO.

2) This investigation revealed that there was more sVSG released from *T. congolense* by interaction with IgM anti-VSG than by interaction with equal amounts of IgG2a anti-VSG. The release of sVSG occurred in an antibody dose-dependent pattern. It was also found that IgM anti-VSG, after interacting with the surface of *T. congolense*, formed soluble immune complexes with released sVSG. The results also showed that antibody-induced release of sVSG can occur without complement, but is enhanced by complement. It was further tested whether fresh sera from either relatively resistant C57BL/6 mice or highly susceptible BALB/c mice, which differ in their complement cascade, had different effects on the release of sVSG from *T. congolense*. The results showed that antibody-induced shedding of sVSG was higher in the presence of fresh C57BL/6 serum than in the presence of fresh BALB/c serum. All these data suggest that the concentration of anti-VSG antibody, antibody class and source of complement can affect the release of sVSG from *T. congolense*.

ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr. Henry Tabel, for his guidance and help throughout my research. I also acknowledge and appreciate the constructive criticisms and invaluable suggestions of members of my advisory committee - Dr. Peter Bretscher, John Gordon, Vikram Misra, Baljit Singh and Lydden Polley. Their contribution to the research project helped in producing a better research work.

I appreciate the assistance of Guojian Wei. He gave me a lot of suggestions during my experiments. I also appreciate Dr. Meiqing Shi's help with the immunocytochemistry. I would also like to thank Mr. Ian Shirley for his help in using Northern Eclipse software. I appreciate Dr. Dele Ogunremi for his help in measuring the band density of western blot results.

I thank my parents, my husband and my son for their loving support. I am grateful for the support of friends - Xiaobei Zhang, Xixing Zhao, Aarti Nayyar, and Aasma Amin. I also thank Oksana Akhova, Noreen Rapin, Phil Gobeil, Pat Thompson and members of staff of the Glassware & Media Preparation Laboratory and Animal Care Unit of the Western College of Veterinary for their help.

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ABBREVIATIONS

CR	Complement receptor
CD	Cluster of Differentiation
DC	Dendritic cell
DEAE	Diethylaminoethyl
DMG	Dimyristoylglycerol
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
F1	First filial generation
F2	Second filial generation
FBS	Fetal bovine serum
GPI	Glycosylphosphatidylinositol
GIP	Glycosyl inositol phosphate
IFN	Interferon
iNOS	Inducible Nitric oxide synthase
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MARCO	Macrophage receptor with collagenous structure

mfVSG	Membrane form of variant surface glycoprotein
MHC	Major histocompatibility complex
NO	Nitric oxide
OD	Optical density
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline containing Tween-20
SDS	Sodium dodecyl sulfate
SE	Standard error
sVSG	Soluble form of variant surface glycoprotein
TC13	*Trypanosoma congolense* clone 13
TH	T helper
TNF	Tumor necrosis factor
TSG	Tris-saline-glucose
VSG	Variant surface glycoprotein

1. LITERATURE REVIEW

1.1 African Trypanosomes

1.1.1 Introduction

African trypanosomes are extracellular parasites causing sleeping sickness in humans and a disease called *nagana* in livestock. The main trypanosome species responsible for the livestock disease are *Trypanosoma congolense, T. vivax* and *T. brucei brucei*. Of these species, *T. congolense* is the most virulent pathogen causing the greatest losses in livestock (Mulligan & Potts, 1970).

Trypanosome species are mainly transmitted to their mammalian hosts by tsetse flies, which inhabit regions between latitudes 14^{o}N and 29^{o}S. About 10 million square kilometers and 37 countries are covered in this tsetse fly "belt", a geographical area equivalent to the combined size of the United States, India and Western Europe (Hursey & Slingenbergh, 1995).

African trypanosomes are responsible for large economic losses in the livestock-based cultures and economies of Africa. The direct production loss only in cattle was estimated to be between US$ 6,000 million to US$ 12,000 million per year (Hursey & Slingenbergh, 1995). If the disease would be controlled, the region could support a further 120 million cattle annually (Nantulya, 1986). Trypanosomiasis control has a long history. Several methods have been employed. The primary approach to disease control is to halt transmission by controlling the insect vectors: the tsetse flies. This has been done by the removal of reservoir hosts (game animals), destruction of tsetse habitat

(bush clearing) and spraying with residual formulations of suitable insecticides (Schofield & Maudlin, 2001). But these measures can result in ecological and environmental problems. Chemotherapy is another measure for controlling trypanosomiasis. The first trypanocidal drug was developed in 1905 (Seed, 2001), but the number of drugs available for the treatment of African trypanosomiasis is very limited. In addition, drug resistance reduces the efficacy of chemotherapy and such resistance is becoming increasingly prevalent (De Koning, 2001, Seed, 2001). Vaccination has been considered to be one of the measures for control, but antigenic variation represents a formidable obstacle that reduces the likelihood of developing an effective VSG-based vaccine. Thus, invariant parasite molecules that are either expressed on the parasite surface or that mediate pathogenesis are being considered as candidates for vaccine design (Taylor, 1998). So far these vaccines only show minimal protection. It is clear that vaccine design has to be based on a better understanding of the immune responses provoked by infection and of the mechanisms involved in either parasite or disease control.

1.1.2 Classification

Trypanosomes are a genus of protozoa belonging to the family Trypanosomatidae, order Kinetoplastida, class Zoomastigophores. The pathogenic trypanosomes belong to the subgenus *Trypanosoma*, and the species of mammalian trypanosomes fall naturally into two major sections characterized by the mode of their development primarily in the vector and secondarily in the mammalian host (Hoare, 1972, Vickerman, 1976)

A. Section Salivaria: The developmental cycle of the salivarian species in the insect-vector is completed in the salivary glands. The parasites are transmitted to the

mammalian host by the bite of the infected insect vector during a blood meal. Most species of this section are important pathogens either in man or domestic animals. This section contains 4 groups: *Duttonella, Nannomonas, Trypanozoon and Pycnomonas*. The constitution of these groups is more homogenous than that of the stercoraria (Hoare, 1972, Vickerman, 1976).

B. Section Stercoraria: The developmental cycle of these species in the insect vector is completed in the fecal medium of the gut, such that transmission occurs by contamination of lesions or wounds produced by scratching the skin in response to irritation of the insect's bite. This section contains three subgroups: *Megatrypanum, Herpestoma and Schizotrypanum* (Hoare, 1972, Vickerman, 1976).

Table 1.1 Classification of mammalian trypanosomes (Hoare, 1972, Vickerman, 1976)

Section	Salivaria	Stercoraria
Subgenus and species	**Subgenus: Duttonella** Species: *Trypanosoma vivax* **Subgenus: Nannomonas** Species: *Trypanosoma congolense*, *T. simiae* **Subgenus: Trypanozoon** Species: *Trypanosoma brucei*, *T. rhodesiense*, *T. gambiense*, *T. evansi* *T. equiperdum* **Subgenus**: **Pycnomonas** Species: *Trypanosoma suis*	**Subgenus: Megatrypanum** Species*: Trypanosoma theileri* **Subgenus: Herpestoma** Species*: Trypanosoma lewisi* and *T. musculi* **Subgenus: Schizotrypanum** Species: *Trypanosoma cruzi*

1.1.3 Morphology

The characterization of African trypanosomes is based on their shape, size, and position of the nucleus, size and location of the kinetoplast, host range and geographical distribution. African trypanosomes are thoroughly adapted to living extracellularly in the blood plasma or tissue fluid of the host. They are elongated and streamlined, tapered at both ends, and range from 8-30 μm in length. A *flagellum* arises at the posterior end from the basal body adjacent to the kinetoplast, and runs the length of the trypanosome. The pellicle, the layer bordering the cytoplasm, while retaining a definite shape, is flexible enough to permit a degree of body movement. Along the length of the body, the pellicle and cytoplasm are pinched up into a thin sheet of tissue called the *undulating membrane* (Hoare, 1972).

T. congolense is a small salivarian trypanosome that is about 13.4 μm to 15.8 μm long (Stephen, 1986). Its kinetoplast, which has important functions in reproduction and metabolism, has a marginal location and is medium-sized (Hoare, 1964). It is sluggish in its movements and has a central nucleus and an undulating membrane (Hoare, 1972, Soltys & Woo, 1987).

1.1.4 Life Cycle

The life cycle of African trypanosomes has two phases, one in the insect vector and another one in the mammalian host.

Transmission is mediated by tsetse flies (*Glossina* species). There are more than 34 species and subspecies of the genus *Glossina* that are recognized at the present time. They belong to three groups: *fusca* group (forest group), *palpalis* group (riverine group),

and *morsitans* group (savannah group). *T. congolense* is capable of developing in all these three groups (Hoare, 1972). The tsetse fly acquires a trypanosomal infection when feeding on an infected mammalian host. The ingested blood stream forms of trypanosomes lose their surface coat in the insect and undergo a cycle of development, in morphologic terms as well as in their metabolism. Ingested trypanosomes change into a longer, slender form called epimastigote. These epimastigotes multiply for about 3 weeks and finally give rise to the infective metatrypanosomes. Metatrypanosomes re-acquire their surface coat of variant surface glycoprotein (VSG) and thus regain infectivity for mammals. This occurs in the salivary glands (Hoare, 1972, Stephen, 1986). The period from ingesting infected blood to the appearance of infective forms in the tsetse fly varies from 7 to 53 days (Hoare, 1972, Nantulya et al., 1978). The tsetse fly remains infective for the remainder of its life once infective metatrypanosomes are present. Infections of the host occur when infected flies bite the host (Molyneux & Ashford, 1983).

At the site of infection, a swelling or chancre may be detected in the skin that is caused by infective metatrypanosomes undergoing development and multiplication. Once the trypanosomes become mature, they are released via lymph vessels and lymph nodes into the blood circulation (Akol & Murray, 1982). Trypanosomes feed by absorbing nutrients from the body fluids of the host, and reproduction occurs through a process of binary division. The parasitemia in the peripheral blood usually becomes apparent in one or two weeks after natural infections. During the growth, trypanosomes vary from long slender forms that predominate in the early logarithmic growth phase of infection to short, stumpy and usually non-dividing forms, which are predominant in the phase of parasite remission (McLintock et al., 1990). The transformation from the long

slender form to the short stumpy form is essential for the vector transmissibility (Seed & Sechelski, 1989).

1.1.5 Trypanosomal antigens

The trypanosomal antigens can be divided into two groups. The first group includes the major surface protein of the bloodstream forms of African trypanosomes, named variant surface glycoprotein (VSG). The VSG form a major component of the 12-15 nm thick electron dense “surface coat” covering trypanosomes. The layers of VSG serve as a protective coat for the parasites. VSG is an immunodominant antigen of the parasite. The major part of the host’s humoral immune response is directed against the VSGs (Cross, 1990), but some of the parasites survive by expressing a different VSG.

The other group is called the non-variable or common antigens. They are the subcellular components, such as structural proteins, enzymes, and membrane constituents (Burgess & Jerrells, 1985, Frommel & Balber, 1987, Shapiro & Murray, 1982). Some of these non-variable antigens may represent cell surface proteins, such as transporters (Parsons & Nielsen, 1990, Webster & Shapiro, 1990), or some enzymes like phospholipase C (Fox et al., 1986, Lamont et al., 1987), trypanosome heat shock protein 60 (HSP60) (Radwanska et al., 2000), and even the C-terminal end of the surface glycoprotein (Rice-Ficht, 1981). These non-variable antigens are expected to remain conserved between trypanosome serodemes, or even different trypanosomatid species or genera (Muller et al., 1992, Olenick et al., 1988).

The non-variant antigens are not exposed to the surface of the intact living trypanosomes, but they are released after parasite lysis by anti-VSG antibodies and

complement. These invariant antigens may play a role in the pathogenesis of the disease. It has been reported that antibody responses to cysteine protease (congopain) are associated with resistance in *T. congolense*-infected cattle (Authie et al., 1993), which suggests that the immune response to this common antigen may play a role in the relative susceptibility or resistance during trypanosome infection.

1.1.6 Antigenic Variation

African trypanosomes will periodically express a different VSG gene that is antigenically distinct from the previously expressed VSG with a switch rate of 10^{-3} to 10^{-5} per cell generation (Cross, 1990). This is called antigenic variation. Each parasite is estimated to have some 1000 distinct VSG genes. The known function of the VSG is to serve as a protective barrier against the attack by the innate immune system on the other outer membrane constituents (El-Sayed et al., 2000, Ferrante & Allison, 1983). *T. congolense* and *T. brucei brucei* that lack surface coats are lysed via the alternative pathway of complement when incubated in normal serum, in contrast to the parasites which possessed a surface coat (El-Sayed et al., 2000, Ferrante & Allison, 1983).

Because of antigen variation, the infected host develops a fluctuating chronic parasitemia and subsequent waves of VSG-specific antibodies. Antigenic variation permits the trypanosome population as a whole to keep “one step ahead ”of the immune response (El-Sayed et al., 2000). The frequent changes of VSG expression during infection and the genetic diversity of the VSGs are thought to represent strategies by African trypanosomes to evade the immune system of their mammalian hosts and to

permit survival of these organisms within the hosts (Kang et al., 2002, Mansfield et al., 2000).

1.1.7 Variant Surface Glycoprotein (VSG)

1.1.7.1 Organization of the VSG Gene

The African trypanosome genome contains about 1000 different VSG genes (Van der Ploeg et al., 1982). Under normal conditions one and only one VSG gene is expressed at a time in a given bloodstream parasite (Baltz et al., 1986, Esser & Schoenbechler, 1985, Munoz-Jordan et al., 1996). The unexpressed VSG genes are scattered among the different chromosomes, including the minichromosomes (Borst et al., 1998, Cross et al., 1998, Donelson, 1996, Pays & Nolan, 1998). In order to be transcribed, VSG genes need to be located in a specialized telomeric environment, which is known as a bloodstream telomere-linked VSG gene expression site (BES). Only a few expressed bloodstream VSG genes have been examined in detail, but all have been found to be transcribed from telomere-linked expression sites that typically consume 45-60 kb, and contain a VSG promoter, a variable number of 70-76 bp repeats, and the VSG gene is followed by subtelomeric and telomeric DNA repeats (Kang et al., 2002). As BESs contain the only active VSG locus in trypanosomes living in the mammalian host, they are the sites of antigenic variation. At any time, only one BES is active and only one VSG gene is transcribed (El-Sayed et al., 2000).

There are two possible mechanisms by which VSG expression can be changed, leading to antigenic variation. They can either switch expression between BESs (telomere conversion) or replace the VSG gene within the active BES (duplicative

transposition). Both mechanisms have been observed (Vanhamme et al., 2001). The molecular events in a given trypanosome that induce transcription at one BES and silence expression at the other BESs are not understood (El-Sayed et al., 2000).

1.1.7.2 Structure of VSG

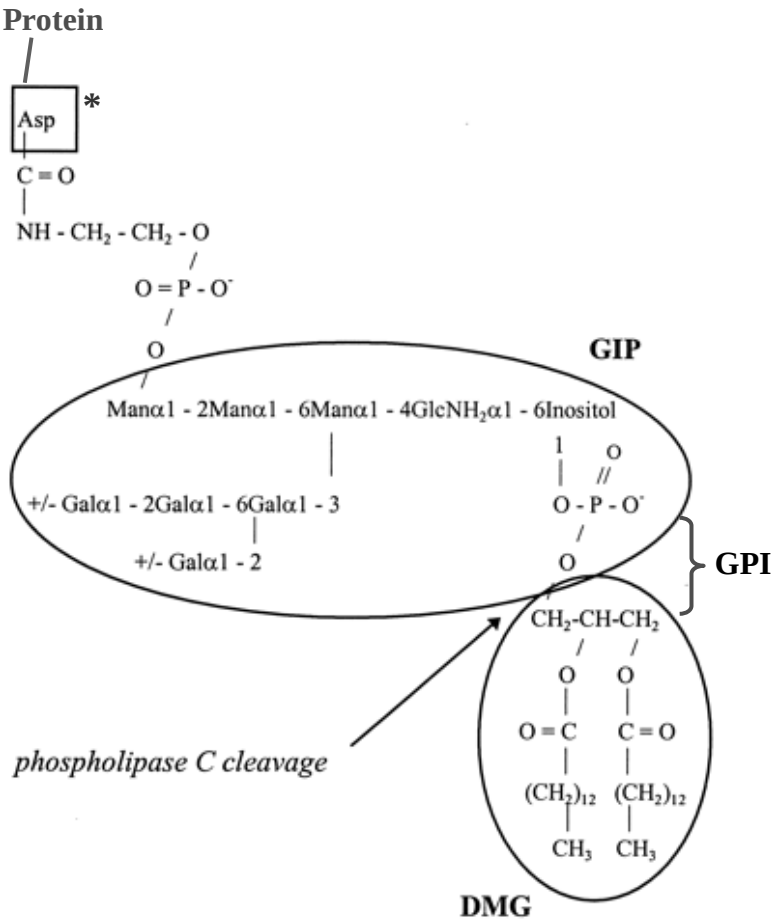
Variant surface glycoprotein (VSG) is the major component of trypanosomes surface protein. VSGs, which cover the entire parasite surface, are arranged in a tightly packed monolayer of homodimers forming a 12-15 nm thick coat which functions as a barrier to lytic serum components but allows nutrients such as glucose to reach transporters in the membrane (Borst et al., 1998). Each parasite expresses 10^7 densely packed identical VSG molecules on their surface (Cross, 1990, Gerold et al., 1996). VSG accounts for about 10% of the total protein and is constitutively synthesized at a high rate (about 8% of total protein biosynthesis) (Kang et al., 2002). This abundant molecule is very immunogenic, and is, therefore, the target of a very potent immune response (Vanhamme et al., 2001).

The molecular weights of VSGs from different species range from 53 to 63 kDa, dependent on the carbohydrate content (Richards, 1984). Tryptic digestion of the C-terminal portion of the VSG molecule can reduce the MW from 53 to 48 kDa (Rosen et al., 1981)

All VSGs may share a similar backbone structure from which emerge distinct epitopes derived from different groups of amino acid side chains. VSGs are all homodimers. Each VSG monomer contains a N-terminal signal sequence and a hydrophobic C-terminal domain and is covalently attached to a glycosylphosphatidylinositol (GPI) membrane anchor (Ferguson et al., 1985, Ferguson

& Williams, 1988, Kang et al., 2002). The outer domain, N-terminal sequence, is highly variable. By X-ray crystallography, the three-dimensional structures of the N-terminal two-thirds of two VSGs have been found to be very similar rod-like shapes despite having quite different amino acid sequences (Blum et al., 1993). The VSG molecules can be cleaved by the trypanosomal GPI-specific phospholipase C (GPI-PLC). This GPI-PLC cleaves the GPI anchor, leaving the dimyristoylglycerol (DMG) compound of the GPI anchored in the membrane, and releases the glycosyl-inositol-phosphate (GIP)-VSG part (also called soluble VSG [sVSG]) (Fox et al., 1986, Magez et al., 1998).

Figure 1.1 The structure of membrane-VSG (mVSG) of *T. brucei* (Magez et al., 1998)



* The boxed Asp is the C-terminal residue of the VSG

1.1.7.3 Release of sVSG

The VSG can be detected in the plasma of rats and mice infected with *T. brucei brucei* (Diffley et al., 1980) and in the plasma of mice infected with *T. congolense* (Tabel unpublished) which indicates that trypanosomes shed surface coat material during the course of infection.

VSG coats are stably associated with the parasite plasma membrane but can be isolated as the membrane form of VSG (mfVSG) (Bulow et al.) or the freely water-soluble glycoproteins (sVSG) after cell lysis. mfVSG is readily converted to sVSG during cell lysis by the action of an endogenous enzyme (GPI-PLC) (Ferguson, 1999). VSG release can be induced by hypotonic lysis (Cardoso De Almeida & Turner, 1983) or by different kinds of stress (Bowles & Voorheis, 1982, Rolin et al., 1996).

GPI-specific phospholipase C (GPI-PLC) is involved in degradation, recycling and shedding of VSG (Carrington et al., 1991, Rolin et al., 1996). Each trypanosome expresses 30,000 copies of a very active GPI-PLC which, by clipping off the dimyristoylglycerol from membrane bound VSG (mfVSG), promotes its shedding in a soluble form (sVSG) (Cardoso De Almeida et al., 1999). It was found that trypanosomal lysates contain a vast abundance of mainly sVSG produced from mfVSG by action of an endogenous GPI-PLC that gains access to surface bound VSG after hypotonic lysis of the cell (Fox et al., 1986). GPI-PLC has been implicated in releasing the VSG coat. However, its role in GPI hydrolysis is still puzzling because (i) GPI-PLC null mutants are fully viable, (ii) cytosolic GPI-PLC is localized away from cell surface VSG, and (iii) GPI-PLC null mutants undergo antigenic variation (Cardoso De Almeida et al., 1999). In vitro experiments showed that shedding of VSG is mediated by GPI hydrolysis, which ultimately accounts for a substantial portion of total release. Surface biotinylation assays

indicate that GPI-PLC does gain access to extracellular VSG. All these results indicate that GPI-PLC plays a significant role in VSG release (Gruszynski et al., 2003). Recent reports also showed that VSG is internalized and recycled exclusively via a specialized part of the plasma membrane, the flagellar pocket. The VSG cell-surface pool is turned over within 12 minutes. VSG recycling happens through endocytosis in large clathrin-coated vesicles that bud from the flagellar pocket membrane, and are delivered to endosomes and/or lysosome. VSG then returns to the cell surface at the flagellar pocket via exocytosis (Engstler et al., 2004).

In the presence of fresh bovine serum, antibodies against *T. congolense* can induce shedding of soluble VSG and the formation of a soluble covalent complex of VSG and bovine complement component C3b (Liu et al., 1993).

1.1.7.4 Effect of VSG on the immune system of the host

GPI-linked variant surface glycoprotein (VSG) represents 10% of the total protein content of trypanosomes (Cross, 1990). Due to the fact that trypanosomes are extracellular parasites, and due to the release of vast amounts of soluble VSG into the circulation, the immune system of an infected mammalian host is regularly confronted with circulating sVSG (Magez et al., 2002). There is evidence that *T. congolense* bloodstream forms evade complement lysis by shedding of immune complexes (Frevert & Reinwald, 1990). It has also been demonstrated that VSG of *T. brucei* cause consumption of complement proteins, which may occur via the tremendous amounts of immune complexes generated during antibody-mediated clearance of each wave of parasitaemia (Musoke & Barbet, 1977). Recent studies have documented the immunostimulatory and regulatory activity of protozoan-derived GPI anchors and

related structures (Ropert & Gazzinelli, 2000). It is clear that GPI-linked macrophage modulation occurs in all protozoan parasite infections (Magez et al., 2002). There is growing evidence that soluble VSG that carries the carbohydrate core (GIP-VSG), after being released from the parasite surface, is involved in affecting functions of macrophages, including the induction of cytokines synthesis, such as TNF-α (Magez et al., 2002, Paulnock & Coller, 2001). TNF-α is a key mediator in trypanosomiasis-associated immunopathology (Hunter et al., 1991, Kang & Schlesinger, 1998, Okomo-Assoumou et al., 1995, Sileghem et al., 1994b). It has also been reported that GIP-VSG inhibits IFN-γ-induced nitric oxide (NO) production by macrophages (Coller et al., 2003). NO has been shown to be trypanostatic for *T. congolense*, *T. musculi*, *T. gambiense* and *T. brucei* in vitro (Kaushik et al., 1999, Vincendeau & Daulouede, 1991, Vincendeau et al., 1992). A complex between VSG and complement component C3b named C3b-VSG has been described (Liu et al., 1993). The C3b-VSG complex is generated when *T. congolense* is incubated with bovine serum in the presence of anti-VSG antibody, and has been suggested to interfere with the phagocytosis of parasites. All these results suggest that sVSG released from parasites may have negative effects on controlling the infection.

1.2 Immunity

African trypanosomes are exposed to host immune system from the moment of injection. One trypanosome is a package of thousands of invariant antigens surrounded by 10 million copies of a single VSG (Vickerman, 1985). The immune system is

continually assaulted by massive amounts of the different VSGs and the invariant antigens.

1.2.1 Innate immunity

Innate immunity refers to antigen-nonspecific defense mechanisms that are designed to recognize highly conserved structures present in many different microorganisms, called pathogen-associated molecular patterns (PAMP) (Aderem & Ulevitch, 2000). The innate resistance to African trypanosomiasis, called trypanotolerance, is manifested by lower parasitemia and less marked anemia (Dargie et al., 1979). The C57BL/6 mouse strain is relatively resistant and the A/J and BALB/c strains are susceptible following infection with *T. congolense*. The speed of control of the first wave of parasites positively correlates with the survival period of mice infected with *T. congolense* (Ogunremi & Tabel, 1995). Macrophages of relatively resistant C57BL/6 mice produce significantly more NO than macrophages of susceptible BALB/c in response to *T. congolense* (Kaushik et al., 1999).. The differences of resistance between C57BL/6 and BALB/c is controlled by five quantitative trait loci (QTL), Tir1, Tir2, Tir3a, Tir3b and Tir3c, positioned on mouse chromosomes 17, 5, 1 respectively (Iraqi et al., 2000). These loci can account for all of the difference between the parental phenotypes but the genes involved in these loci and their mode of mechanism are presently unknown.

1.2.1.1 Role of complement

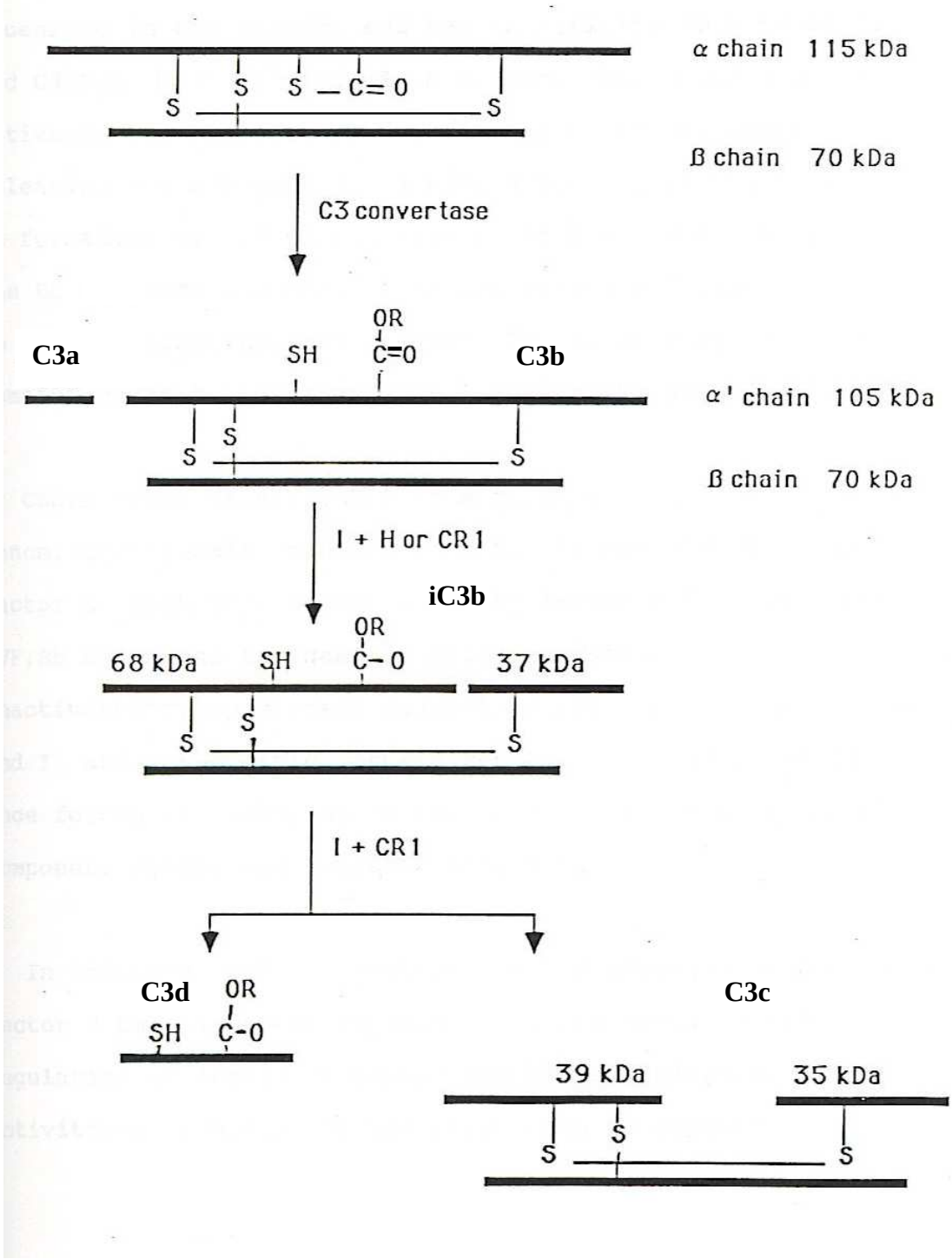
Complement, first described in the 1890s, is a major defense and clearance system in the bloodstream. It can be activated via antibody-antigen complex (classical pathway) or directly via a foreign particle or organism (alternative pathway). It is an important part of the host's immune system that functions together with the adaptive immune response to provide effective mechanisms: killing microorganisms by lysis, facilitating the clearance of microorganisms as well as immune complexes by phagocytosis, and initiating inflammation (Law & Reid, 1988).

Complement-mediated lysis of trypanosomes can be observed in *vitro* (Balber et al., 1979) and in *vivo* (Vickerman & Barry, 1982). Trypanosomal infections in cattle are accompanied by a severe depression of hemolytic complement activity (Nielsen et al., 1978, Tabel et al., 1980) that is less profound in sera from trypanotolerant cattle than that from susceptible ones (Authie & Pobel, 1990). Complement depletion in the serum could cause further defects in the functioning of the immune response, such as an increased susceptibility of trypanosome-infected animals to secondary infections (Maxie et al., 1979, Naessens et al., 2000).

The complement system is composed of about 30 proteins. Of all complement components, the component C3 (M.W. = 185kD) is the most prevalent plasma glycoprotein (present at a concentration of 1.3 mg/ml). It plays a central role in the system since the classical and the alternative pathways merge at the C3 activation step (Law & Reid, 1988). Activation of complement component C3 leads to either humoral cell killing via assembly of the membrane attack complex, or phagocytosis of the opsonized target via the complement receptors on macrophages (Ross & Auger, 2002).

C3 is composed of an α chain with molecular weight of 115 kD linked with a 70 kD β chain by several disulfide bonds (Morris et al., 1982).

Figure 1.2 Degradation of complement C3 (Adapted from (Hostetter & Gordon, 1987))



C3 can undergo proteolytic cleavage into C3a and C3b in the classical pathway, induced by the complex of C4b2a, or be cleaved by serum proteases in the alternative pathway. C3b can covalently bind to carbohydrate of foreign cell surfaces. Bound C3b is rapidly converted to iC3b by serum factor I and its cofactors (Law & Levine, 1977, Law et al., 1979). It has also been reported that iC3b has a much longer half-life than C3b in blood (Cain et al., 1987, Ross, 1986). The digestion of iC3b to C3c and C3dg by serum proteases is relatively slow. Hence, the majority of bound C3 fragments on target surfaces is in the form of iC3b (Law & Reid, 1988). It is well established that the presence of iC3b on pathogens enhances their phagocytosis by macrophages and polymorphonuclear neutrophils by interacting with complement receptor 3 (CR3) (Cain et al., 1987, Preynat-Seauve et al., 2004, Rosenthal et al., 1996, Ross & Medof, 1985). Homogenates of *T. congolense* (Tabel, 1982) or trypanosomes lacking a glycoprotein coat can activate the alternative complement pathway (Ferrante & Allison, 1983). It has been proposed that during an infection, the binding of IgM antibody to trypanosomes results in the deposition of complement component C3 cleavage products, such as C3b or iC3b, on the parasites (Devine et al., 1986). The opsonized parasites could thus interact with Kupffer cells via specific complement receptors.

1.2.1.2 Macrophages

1.2.1.2a Biology of macrophages

Macrophages are widely distributed throughout the body. They can be found in the liver (Kuffer cells), lymphoid organs, spleen (macrophage of the marginal zones and red pulp macrophages), lungs (alveolar macrophages), central nervous system, serous cavities, bone, synovium, skin etc. They can participate in a wide range of physiological

and pathological processes (Ross & Auger, 2002). They differentiate from the mononuclear phagocyte system. This system comprises bone marrow monoblasts and promonocytes and peripheral blood monocytes (Ross & Auger, 2002). Precursors of macrophages originate in the bone marrow. The monoblast is the least mature cell of the mononuclear phagocyte system, with each giving rise to two promonocytes, the direct precursors of monocytes. Each dividing promonocyte gives rise to two monocytes. Monocytes remain in the bone marrow for less than 24 h before entering the peripheral blood. The peripheral blood monocytes migrate into extravascular tissues to become macrophages. The proportion of monocytes migrating to various organs is apparently random and corresponds roughly to the size of the organ (Van Furth & Cohn, 1968). The liver contains about 80% of the total macrophage population of a mouse (Knolle & Gerken, 2000). The tissue macrophages remain in the tissues for several months and do not return to the circulation. Macrophages in tissues and body cavities are not a constant population of cells, but are being renewed regularly by the influx of monocytes (Ross & Auger, 2002).

Macrophages are generally large, irregularly shaped cells measuring 25-50 μm in diameter. They often have an eccentrically placed, round or kidney-shaped nucleus with prominent nucleoli and finely dispersed nuclear chromatin. Their cytoplasm contains both fine granules and large azurophilic granules and a clearly defined juxtanuclear Golgi complex, as well as clear cytoplasmic vacuoles (Goldman, 1989). Macrophages represent a major defense against invasion of the host by a wide variety of microorganisms, including bacteria, viruses, fungi and protozoa (Ross & Auger, 2002).

1.2.1.2b Function of macrophages

Macrophages count among the most pleiotropic cells of the immune system, exhibiting a plethora of biological functions, including phagocytosis, killing of invading microorganism and pro- and/or anti-inflammatory activities. They participate in adaptive responses through production of a variety of cytokines, expression of costimulatory molecules and antigen presentation by their class I and II MHC expression (Noel et al., 2002). Several functions of murine macrophages are activated during infections by African trypanosomes, including phagocytosis, adherence, pinocytosis, oxidative burst, prostaglandin release and cytokine secretion (Sileghem et al., 1994a). The elimination of trypanosomes by antibodies is thought to be predominantly mediated by opsonization and destruction by liver macrophages, rather than by complement-mediated lysis (Dempsey & Mansfield, 1983, Jokiranta et al., 1995). Anti-VSG antibody-mediated phagocytosis of trypanosomes by macrophages is considered the most important mechanism of clearance of trypanosomes from the blood of infected mice (Macaskill et al., 1980, Shi et al., 2004). Although there are apparently no differences at the level of phagocytosis of trypanosomes, macrophages of susceptible and resistant mice differ in their physiological responses after phagocytosis of *T. congolense* (Kaushik et al., 1999, Tabel et al., 2000). Peripheral blood mononuclear cells and monocytes of resistant N'Dama cattle were found to produce more nitric oxide (NO) but less IL-10 than those of susceptible Boran cattle (Taylor et al., 1998). A similar result was shown in the mouse model. Macrophage of resistant C57BL/6 produce significantly higher amounts of NO than macrophages from susceptible BALB/c mice in response to *T. congolense* (Kaushik et al., 1999). It was also demonstrated that different antibody classes might

activate macrophages via different signaling pathways. Previous studies showed that phagocytosis of *T. congolense* mediated by anti-VSG antibodies of IgG2a isotype in the presence of IFN-γ induced two- to nine-fold more NO than phagocytosis mediated by IgM antibodies by bone marrow-derived macrophages (BMDM) from both C57BL/6 and BALB/c mice (Kaushik et al., 1999). It has been well documented that IgG-mediated phagocytosis occurs via Fc receptors and macrophages activated via Fc receptors can produce large amounts of oxidative burst products, such as NO (Bayon et al., 1997, Becherel et al., 1995, Dasgupta et al., 2000, Kolb et al., 1995, Vouldoukis et al., 2000). The macrophage receptors involved in IgM-mediated phagocytosis of trypanosomes are presently not known.

1.2.1.3 Role of nitric oxide

NO, a free radical gas, works as a nonspecific cytotoxin in the host immune system (MacMicking et al., 1997, Moilanen & Vapaatalo, 1995). It is small and lipophilic which makes it easy to enter microbes. The prime targets of NO are sulfhydryls and iron which are central in the biochemistry of microbes (MacMicking et al., 1997). Nitric oxide (NO) produced by activated macrophages in response to various stimuli or pathogens is considered to be an important arm of nonspecific immunity (May & Machesky, 2001).

Sustained production of NO endows macrophages with cytostatic or cytotoxic activity against viruses, bacteria, fungi, protozoa, helminthes, and tumor cells (Anggard, 1994, Snyder & Bredt, 1992). Different stimuli or pathogens can use different signaling pathways for NO production. Previous studies showed that Fc receptors (Bayon et al., 1997, Becherel et al., 1995), Toll-like receptors (Mizel et al., 2003) and mannose

receptors (Karaca et al., 1995) are involved in inducing NO synthesis. The high-output NO pathway has probably evolved to protect the host from infection (MacMicking et al., 1997). NO has been shown to be trypanostatic for *T. congolense*, *T. musculi*, *T. gambiense* and *T. brucei* in vitro (Kaushik et al., 1999, Vincendeau & Daulouede, 1991, Vincendeau et al., 1992). Peritoneal macrophages and bone marrow-derived macrophages of resistant C57BL/6 mice produce significantly higher NO than macrophages of susceptible BALB/c in response to *T. congolense* (Kaushik et al., 1999). NO has also been reported to be partially responsible for macrophage-mediated splenic immunosuppression observed in *T. brucei*-infected highly susceptible mice (Mabbott et al., 1995), but not in *T. congolense*-infected mice (Uzonna et al., 1998c). The role of NO in relatively resistant mice remains to be determined. Recent reports show that NO contributes to the immunosuppressive activity only during the early stages of murine *T. brucei* infection (Beschin et al., 1998). NO was not upregulated during *T. congolense* infection of cattle and was not found responsible for reduction in T cell proliferation (Taylor, 1998, Taylor et al., 1996). Therefore, the role of NO in trypanosome infection still needs to be determined, but it may at least partially account for differences in resistance to *T. congolense* infection in mice.

1.2.2 Adaptive immunity

Adaptive (acquired) immunity, including two major categories (humoral immunity and cell-mediated immunity), refers to antigen-specific defense mechanisms.

1.2.2.1 Humoral immune responses

Humoral immunity is mediated by antibody molecules in response to an antigen and they are produced by B-lymphocytes. The VSG surface epitope-specific B-cell responses in trypanosome-infected mice represent composite T-cell-independent and T-cell-dependent processes (Reinitz & Mansfield, 1990). The T cell-independent, VSG-specific, B-cell responses are associated with temporary immunity to the trypanosome variant antigenic types (VATs) arising during chronic infection (Campbell & Phillips, 1976, Pinder et al., 1986, Reinitz & Mansfield, 1990). The relative contribution of T-cell-independent and T-cell-dependent processes to the total VSG-specific antibody produced during infection is variable, which may reflect differences in the primary structure or display of VSG molecules on the trypanosome membrane or may represent active parasite interference with some epitope-specific B cell responses (Reinitz & Mansfield, 1990).

The B-cell specific responses to the trypanosomal variant surface glycoprotein (VSG) result in the elimination of organisms expressing the target surface antigen, and thereby control the parasitemia. Parasites are rapidly eliminated from the circulation in the presence of specific antibody (Mansfield, 1990, Shi et al., 2004). The mice that are highly susceptible to trypanosomiasis produce little or no detectable antibody against the VSG during an infection (Black et al., 1983, Morrison & Murray, 1985) in spite of their B lymphocytes being extensively activated while resistant mice exhibit superior antibody synthesis and secretion (Black et al., 1986, Levine & Mansfield, 1984, Newson et al., 1990). The primary immune response to the VSG reaches a maximum within 7-14 days after challenge (Roelants & Pinder, 1984) and consists of both IgM and IgG classes of antibody. IgM and IgG antibodies against VSG appear equally effective in mediating

phagocytosis in vitro. However, IgM has been shown to be the only class detectable during the initial parasitemic wave (Dempsey & Mansfield, 1983). It also has been shown that the highly immunogenic VSG can induce high levels of IgM antibodies in infected cattle and mice (Radwanska et al., 2000). The enormous IgM antibody production is not accompanied by a concomitant increase in IgG antibody in susceptible BALB/c mice (Uzonna et al., 1999). Whether the type of antibody response is the cause for resistance to the infection as measured by survival time is still not clear, but differences in the levels of antibody production and in the class of antibody produced have been found between relatively resistant and susceptible mice.

1.2.2.2 Role of antibody

The production of antibody to the various predominant VSGs expressed provide protective immunity in infected animals (Naessens et al., 2000). VSG-specific antibodies mediate complement-mediated lysis (Crowe et al., 1984, Flemmings & Diggs, 1978), agglutination (Russo et al., 1994), immobilization (Wei et al., 1990) and increased uptake by macrophages (Ngaira et al., 1983, Shi et al., 2004) of trypanosomes. It is established that the clearance of the predominant variant antigenic type of parasite is an immune-mediated mechanism and requires VSG-specific antibodies (Pinder et al., 1986). Opsonization with VSG-specific antibodies results in rapid uptake of the parasites by macrophage (Radwanska et al., 2000, Shi et al., 2004, Uzonna et al., 1999). In the absence of antibodies, bloodstream trypanosomes are protected against phagocytosis by the surface coat (Mosser & Roberts, 1982). Antibodies to non-variant antigens may not be involved in killing or clearance of trypanosomes. They, however, may neutralize the

pathogenic or toxic effects of certain trypanosomal molecules, and therefore, may prevent anemia after repeated heterologous infections (Paling et al., 1991).

It was found that, upon infection, highly susceptible BALB/c mice made IgM antibodies to common trypanosomal epitopes earlier than relatively resistant C57BL/6 mice. In contrast to C57BL/6 mice, BALB/c mice failed to switch to produce IgG3 and IgG2a antibodies (Uzonna et al., 1999). When infected BALB/c mice were cured by treatment with Berenil, they did produce antibodies of IgG3 and IgG2a isotypes reacting with common trypanosome epitopes. Mice that underwent self-cure after *T. congolense* infection were found to have the highest serum levels of IgG3 and IgG2a antibodies to common antigens of *T. congolense* (Uzonna et al., unpublished). It has been observed that monoclonal IgG2a anti-VSG plus *T. congolense*, when added to macrophage cultures, induced 2 to 9-fold more NO in the macrophages than those trypanosomes opsonised with monoclonal IgM anti-VSG antibody (Kaushik et al., 1999). IgM was more efficient than IgG in agglutination, and complement-mediated lysis of *T. brucei* (Seed, 1977). In vitro experiments showed that IgM and IgG anti-VSG have equivalent effects in regard to phagocytosis, but they have different effects on macrophage activation (Kaushik et al., 1999).

1.2.2.3 Cellular immune responses

After trypanosome infection, T-lymphocyte proliferation occurs but is depressed as infection progresses (Mayor-Withey et al., 1978). The T cell responses against trypanosomes are not well characterized. T-cell requirements were examined by analysis of gamma globulin preparations derived from trypanosome-infected BALB/c nude

(nu/nu) and thymus-intact (nu/+) mice. Analysis of VSG-specific antibody in infected mice demonstrated that in the absence of T cells there was a significant B-cell response to exposed VSG epitopes; however, in the presence of T cells these surface epitope-specific responses were greatly enhanced. Immunization with VSG in the absence of infection elicited only T-cell-dependent responses (Reinitz & Mansfield, 1990). The first direct evidence of stimulation of VSG-specific Th cells during trypanosome infection was obtained by Schleifer (Schleifer & Mansfield, 1993). CD4+ Th cells specific for VSG were found predominantly in the peritoneal cavity of infected resistant mice, but TH cell responses in infected susceptible mice were not studied (Schleifer & Mansfield, 1993). The immunity is not transferred with T cell-enriched spleen subpopulations. It was shown that treatment of spleen cells with anti-thymic cell and complement did not affect their ability to transfer immunity (Takayanagi & Nakatake, 1975). Mice deprived of B cells, but not T cells, are unable to mount an effective response (Campbell et al., 1977). Reduced T and B cell responsiveness has been attributed to T-cell-mediated immunosuppression (Eardley & Jayawardena, 1977, Jayawardena et al., 1978, Pearson et al., 1978, Pearson et al., 1979). Recently reports showed that there are suppressor (Uzonna et al., 1998c) and pathogenic T cell populations (Shi et al., 2003) in *T. congolense*-infected mice. These cells are the major producers of IFN-γ, which contributes to the suppression of splenocyte proliferative responses to Con A in the mice (Uzonna et al., 1998a, Uzonna et al., 1998b). These cells are also involved in the *in vitro* suppression of B cell responses to a T cell-dependent Ag as well as in the suppression of T cell proliferative responses to Con A. It appears that the IFN-γ producing T cells that

show suppressor activity and disease-enhancing effects are predominantly $CD4^+$ (Shi et al., 2003, Uzonna et al., 1998c) and MHC-II-restricted (Shi, unpublished).

1.2.2.4 Role of cytokines

IFN-γ is an important cytokine in the pathogenesis of trypanosomiasis. Enhanced induction and secretion of IFN-γ during *T. congolense* infections mediates mortality in highly susceptible BALB/c mice (Shi et al., 2003, Uzonna et al., 1998a). Treatments of infected susceptible BALB/c mice with anti-IFN-γ antibody significantly reduces the parasitemia of these highly susceptible mice and increases their life-span (Shi et al., 2003, Uzonna et al., 1998a). *T. brucei* infected mice with disrupted IFN-γ genes have been reported to have decreased parasitemia and increased survival times (Bakhiet et al., 1996). The opposite results have also been observed (Hertz et al., 1998, Namangala et al., 2001). IFN-γ has been claimed to act as a growth factor for *T. brucei* (Olsson et al., 1991). However, IFN-γ was not found to have growth stimulatory effects on *T. congolense* (Kaushik et al., 1997). IFN-γ can play a detrimental or beneficial role in animals infected with African trypanosomes. The role of IFN-γ may depend on a number of factors, such as the trypanosome species, virulence of the infecting strains, as well as the genetic status of the trypanosome-bearing host.

Tumor necrosis factor (TNF-α) is an inflammatory cytokine which exerts a wide range of biological activities including the induction of cellular proliferation and differentiation, apoptosis, cytotoxicity, inflammation and immunomodulation (Aggarwal & Natarajan, 1996). Induction of TNF-α and its role in immunopathology has been extensively documented in African trypanosomiasis (Hunter et al., 1991, Kang &

Schlesinger, 1998, Okomo-Assoumou et al., 1995, Sileghem et al., 1994b). Enhanced expression of TNF-α in the brains of *Trypanosoma brucei*-infected mice has been reported (Hunter et al., 1991). The continual release of TNF-α (cachectin) from activated macrophages are the hallmark of chronic wasting disease associated with trypanosomiasis (Beutler & Cerami, 1988). Associations have also been found between TNF-α production by monocytes and the severity of disease-associated anemia in trypanosome-infected cattle (Sileghem et al., 1994b), the neuropathological symptoms in human sleeping sickness patients (Okomo-Assoumou et al., 1995) and trypansosome-elicited immunosuppression and overall morbidity (Magez et al., 1999). There are no reports of TNF-α playing a role in resistance to *T. congolense* infections in mice (Magez et al., 1999).

IL-10 is known to suppress expression of other cytokines and regulatory molecules, such as NO (Taylor et al., 1998). *T. congolense*-infected cattle have elevated levels of mRNA encoding IL-10 in cells from blood, lymph node and spleen, suggesting that IL-10 might be related to the apparent failure of bovine monocytes to produce inflammatory molecules (Taylor et al., 1998). The increase in IL-10 is found in both tolerant N'Dama and susceptible Boran cattle and hence may not be associated with trypanotolerance or susceptibility (Naessens et al., 2000). Following infection with *T. congolense*, the plasma of the highly susceptible BALB/c mice contains significantly higher levels of IL-10 than that of relatively resistant C57BL/c mice. Anti-IL-10 antibody treatment significantly reduced the parasitemia and moderately increased the survival period of highly susceptible BALB/c mice (Uzonna et al., 1998b), which suggests that IL-10 is a disease-enhancing cytokine during *T. congolense* infection in

mice. On the other hand, IL-10 is absolutely required to counteract the detrimental effects of IFN-γ in *T. congolense*-infected mice (Shi et al., 2003).

1.3 Receptors on macrophages involved in phagocytosis

Various receptors have been reported on the surface of macrophages. They control multiple of activities of macrophages, such as growth, differentiation, activation, endocytosis, recognition, migration and secretion (Ross & Auger, 2002).

Phagocytosis, a phrase first coined by Metchnikoff (cited by (Ross & Auger, 2002)), is defined as the cellular engulfment of large particles, usually those over 0.5 μm in diameter (May & Machesky, 2001). Phagocytosis of pathogens by macrophages can initiate innate immune responses, which in turn orchestrates the adaptive response. In order to discriminate between infectious agents and self, macrophages have evolved a restricted number of phagocytosis receptors (Aderem & Underhill, 1999).

1.3.1 Fc receptors

The receptors for the Fc region of the IgG molecule are one kind of receptor that was firstly identified on the macrophages (Berken & Benacerraf, 1966). The attachment of the Fc portions of immunoglobulin (Igs) to the surface of the macrophage via Fc receptors triggers various functions, such as endocytosis, transmembrane signal generation, phagocytosis, and secretion of potent mediators (production of superoxide, cytokines etc.) (Roitt, 1994, Ross & Auger, 2002). Antibodies act as opsonins that render the particle they coat more susceptible to engulfment by phagocytic cells. Most of the understanding of the signaling pathways leading to phagocytosis in macrophages

comes from studies involving the Fc receptor (Ravetch, 1997, Ravetch & Clynes, 1998, Unkeless et al., 1995).

There are different Fc receptors on macrophages that can recognize Fc portions of IgG (FcγRs) and IgA (FcαR) (Ross & Auger, 2002). A range of FcγRs exist (Sanchez-Mejorada & Rosales, 1998). FcγRI (Indik et al., 1994), FcγRIIA (Tuijnman et al., 1992) and FcγRIIIA (Park et al., 1993) can all support phagocytosis in human macrophages. FcγRI (CD64) has high affinity for monomeric IgG, while FcγRII (CD32) and FcγRIII (CD16) have only low affinity for monomeric IgG, but can effectively bind immune complexes by multiple receptor-ligand interactions (Unkeless et al., 1988). There is no mouse counterpart of the human FcγRIIA (Aderem & Underhill, 1999). Murine and human macrophages express FcγRIIB (an isoform of FcγRIIA), an inhibitory receptor that negatively regulates phagocytosis (Hunter et al., 1998, Odin et al., 1991, Ravetch, 1994). FcγRIIIB is able to initiate calcium signaling and actin polymerization, but its role in phagocytosis remains unclear (Chuang et al., 2000, Kimberly et al., 1990, Salmon et al., 1991).

FcγRI and FcγRIIA are expressed on neutrophils and macrophages, which are both professional phagocytes. FcγRIIIA is restricted to macrophages, and FcγRIIIB is present only on neutrophils (Sanchez-Mejorada & Rosales, 1998). FcγR-mediated ingestion occurs by a zipper-like process, in which FcγRs in the macrophage plasma membrane interact sequentially with IgG molecules distributed over the surface of the ingested particle (Silverstein, 1995). FcγR-induced phagocytosis is tightly coupled to the production and secretion of proinflammatory molecules such as reactive oxygen intermediates and proinflammatory cytokines (Aderem et al., 1985, Wright et al., 1983).

1.3.2 Complement receptors

The receptors for the cleavage products of the third component of complement (C3) are other receptors on macrophages (Lay & Nussenzweig, 1968). Activation of the complement component C3 can lead either to humoral cell killing via assembly of the membrane attack complex or to phagocytosis of the opsonized target via the complement receptor (Ross & Auger, 2002). Complement-receptor-mediated phagocytosis is morphologically distinct from that mediated by FcRs, although both processes require actin polymerization. Complement-opsonised particles 'sink' into the phagocyte; there is minimal membrane disturbance, and internalization does not usually lead to an oxidative burst (May & Machesky, 2001).

Complement proteins opsonize particles for phagocytosis by the C3b or iC3b receptors (CRs) on macrophages. Receptors expressed on macrophages that participate in phagocytosis of complement-opsonized particles, include CR1, CR3 and CR4 (Carroll, 1998, Sengelov, 1995). CR1 is a single chain transmembrane protein consisting of a large extracellular lectin-like complement-binding domain and a short 43 amino acid cytosolic domain. CR1 binds C3b and primarily functions in particle adherence rather than internalization (Brown, 1991). CR3 and CR4 are integrin family members made up of heterodimers of different α chains (CD11b for CR3 and CD11c for CR4) noncovalently associated with a shared β chain (CD18). CR3 molecules bind specifically to iC3b and are responsible for particle internalization. The ligand for CR4 is still unclear but may also be iC3b (Sengelov, 1995).

CR3 has been the most widely studied. It is predominantly expressed on neutrophils, monocytes, macrophages, and natural killer cells (Arnaout, 1990). Distinct

functional domains have been identified in the extracellular portion of the CD11b subunit of CR3 (Balsam et al., 1998, Dana et al., 1986, Diamond et al., 1993, Lu et al., 1998, Thornton et al., 1996): the I- or A- domain is essential for binding and phagocytosis of iC3b-coated particles. CR3 is capable of binding to several ligands through different recognition sites. Binding to iC3b leads to particle phagocytosis, but the receptor can also mediate non-opsonic phagocytosis of particles by binding to molecules such as β-glucan (Thornton et al., 1996). The phagocytosis of iC3b-opsonised particles by CR3 proceeds efficiently only if the phagocyte is first activated, for example, by inflammatory cytokines or by attachment to the extracellular matrix (Brown, 1986, Pommier et al., 1983). Notably, CR3 has been shown to associate with FcγRIII (Zhou et al., 1993), and the two phagocytic receptors cooperate in regulating the respiratory burst (Zhou & Brown, 1994).

1.3.3 Other receptors

1.3.3.1 Mannose receptors

The mannose receptor (MR) on macrophages recognizes mannose and fucose on the surface of pathogens and mediates phagocytosis of the organisms (Stahl & Ezekowitz, 1998). MR is a phagocytic receptor with broad pathogen specificity because of its high affinity for branched mannose and fucose oligosaccharides (Aderem & Underhill, 1999). It is perhaps the best characterized of the macrophage lectins involved in host defense. MR is a 175-KDa single chain receptor with a short cytoplasmic tail and an extracellular domain including 8 lectin-like carbohydrate-binding domains. The cytoplasmic tail is crucial to both the endocytic and phagocytic functions of the receptor,

but little is known about the signals that lead to phagocytosis (Ezekowitz et al., 1990, Stahl & Ezekowitz, 1998).

The expression of the MR is regulated (Rouleux-Bonnin et al., 1994, Rouleux-Bonnin et al., 1995). Although previously it was thought that it was restricted to tissue macrophages, it is clear that the MR is expressed on immature dendritic cells (DCs) (Reis e Sousa et al., 1993, Sallusto et al., 1995), subsets of endothelial cells (Bijsterbosch et al., 1996, Magnusson & Berg, 1993), tracheal smooth muscle cells (Lew & Rattazzi, 1991, Lew et al., 1994), retinal pigment epithelium (Lutz et al., 1995, Shepherd et al., 1991), kidney mesangial cells (Liu et al., 1996), and Kaposi sarcoma cells (Uccini et al., 1997). MR expression is modulated by immunoglobulin receptors (Schreiber et al., 1991a, Schreiber et al., 1991b), pathogens and their products (Lefkowitz et al., 1997, Shepherd et al., 1997) and other factors (Schreiber et al., 1990).

The mannose receptor recognizes the patterns of carbohydrates that decorate the surfaces and cell walls of infectious agents, and has been implicated in binding and phagocytosis of a wide range of microorganisms, such as Gram-negative and Gram-positive bacteria, yeast, parasites and mycobacteria (Linehan et al., 2000). An important role of MR in host defense is suggested by the fact that MR-mediated phagocytosis is modulated by the proinflammatory cytokine IFN-γ (Marodi et al., 1993). During IFN-γ-mediated macrophage activation, receptor expression is downregulated as a result of reduction in the MR mRNA transcripts. However, IFN-γ-treated macrophages exhibit an increased capacity to kill the yeast *Candida albicans* in a MR-dependent manner (Marodi et al., 1993). It appears that the MRs that remain accessible after IFN-

γ treatment significantly enhance MR-dependent phagocytosis (Marodi et al., 1993, Stahl & Ezekowitz, 1998).

1.3.3.2 Scavenger receptors

Scavenger receptors (SRs) are a family of cell surface glycoproteins able to bind modified low density lipoprotein (mLDLs) (Krieger, 1997). A number of different scavenger receptors (SR-A, SR-B SR-C, SR-D, SR-E, SR-F, SR-PSOX, FEEL-1 AND FEEL-2) have now been identified, not only in macrophages but also other cells. The growing scavenger receptor family also currently includes cell surface receptor (SR-BI) whose main ligand is high density lipoprotein (HDL) (Horiuchi et al., 2003). SRs can alter cell morphology and their expression is affected by various cytokines (Peiser et al., 2002).

SRs have several functions, including endocytosis, antigen presentation, adhesion and phagocytosis and pattern recognition. They play an important role in uptake and clearance of effete components, such as modified host molecules and apoptotic cells. SRs can also bind and internalize microorganisms and their products, such as Gram-positive bacteria, Gram-negative bacteria, intracellular bacteria and CpG DNA (Peiser et al., 2002). SR-A-mediated phagocytosis of apoptotic cells (Platt et al., 1996), and Gram-negative and Gram-positive bacteria has been demonstrated (Thomas et al., 2000). The cell biology of SR-A-mediated phagocytosis following receptor ligation is not understood and the response of macrophages following SR-A-mediated ingestion of both apoptotic cells and microorganisms has not been investigated. Macrophage receptor with collagenous structure (MARCO) can also bind Gram-negative and Gram-positive

bacteria. Although no data directly support that it is involved in phagocytosis, MARCO is expressed on macrophages of the marginal zone in the spleen, a population which is highly phagocytic and efficient at capturing antigens from the blood stream (Peiser & Gordon, 2001).

1.3.3.3 Toll-like receptors

Toll-like receptors (TLRs) have been identified as ancient receptors that confer specificity to the host's innate immune system allowing the recognition of 'pathogen-associated molecular patterns (PAMPs) (Aderem & Ulevitch, 2000, Kopp & Medzhitov, 1999). In mammals there are at least 10 members of the Toll-like receptor (TLR) family (TLR1-TLR10) that recognize specific components conserved among microorganisms (Takeda et al., 2003). TLR family members are characterized structurally by the presence of a leucine-rich repeat (LRR) domain in their extracellular domain and a TIR (Toll/IL-1) domain in their intracellular domain (Takeda et al., 2003). TLRs are required for detection of a broad range of microbial products including lipopolysaccharide, peptidoglycan, and bacterial lipopeptides (Aderem & Ulevitch, 2000, Akira et al., 2001), and several TLR family members are actively recruited to phagosomes during microbe internalization (Ozinsky et al., 2000, Underhill et al., 1999a). Expression of TLRs is modulated by a variety of factors such as microbial invasion, microbial components, and cytokines (Takeda et al., 2003).

TLR2 recognizes components from a variety of microorganisms, including lipoproteins from pathogens such as Gram-negative bacteria, Mycoplasma and spirochetes (Aliprantis et al., 2000, Brightbill et al., 1999, Hirschfeld et al., 1999, Lien et al., 1999), peptidoglycan and lipoteichoic acid from Gram-positive bacteria (Lehner et

al., 2001), lipoarabinomannan from mycobacteria (Underhill et al., 1999b), a phenol-soluble modulin from *Staphylococcus epidermidis* (Hajjar et al., 2001), zymosan from fungi (Underhill et al., 1999a), glycolipids from *Treponema maltophilum* (Opitz et al., 2001), porins that constitute the outer membrane of *Neisseria* (Massari et al., 2002), and also glycoinositolphospholipids from *Trypanosoma cruzi* (Campos et al., 2001). The GPI anchors purified from *T. cruzi*, which contain a long glycan core and unsaturated fatty acids, triggered TLR-2 at subnanomolar concentrations (Campos et al., 2001). By using TLR-2 knock out mice, it was found that TLR-2 expression appears to be essential for induction of IL-12, TNF-α and NO by GPI anchors, which means that GPIs are potent activators of TLR-2 from both mouse and human origin. The activation of TLR-2 may initiate the host's innate defense mechanisms and inflammatory response during protozoan infection. TLR6 functionally cooperates with TLR2 to recognize microbial lipopeptides (Takeuchi et al., 2001). TLR2 and TLR6 co-immunoprecipitate, suggesting that they physically interact in the cell (Ozinsky et al., 2000).

2. HYPOTHESES AND OBJECTIVES

2.1 Hypotheses

1. CR3 (Mac-1, CD11b/18) is involved in IgM anti-VSG mediated phagocytosis of trypanosomes
2. Monoclonal IgM anti-VSG induces shedding of soluble VSG from *T. congolense*

2.2 Objectives

1. Test whether CR3 (Mac-1; CD11b/18) is involved in IgM anti-VSG-mediated phagocytosis of *T. congolense* by macrophages
2. Test the effects of anti-VSG antibody and complement on the release of soluble VSG from *T. congolense*

NOTE:

Chapter 3 was submitted on Feb, 16, 2005 to Journal of Infection and Immunity as: Wanling Pan, Oladele Ogunremi, Guojian Wei, Meiqing Shi, and Henry Tabel, CR3 (CD11b/CD18), the major macrophage receptor for IgM antibody-mediated phagocytosis of *Trypanosoma congolense*: diverse effect on subsequent synthesis of TNF-α and nitric oxide.

The work was all done by Wanling Pan, except the data for Figure 3.1 and Figure 3.7 that were produced by Dele Ogunremi.

3. CR3 (CD11b/CD18), THE MAJOR MACROPHAGE RECEPTOR FOR IGM ANTIBODY-MEDIATED PHAGOCYTOSIS OF *TRYPANOSOMA CONGOLENSE*: DIVERSE EFFECT ON SUBSEQUENT SYNTHESIS OF TNF-α AND NITRIC OXIDE

3.1 Abstract

Production of antibodies to the variant surface glycoprotein (VSG) of African trypanosomes is one of the major immune responses leading to control of parasitemia. IgM antibodies are the first and predominant class of anti-trypanosomal antibodies in infected animals. IgM antibodies to VSG (IgM anti-VSG) mediate phagocytosis of *Trypanosoma congolense*. The receptor(s) on macrophages that recognizes IgM anti-VSG-coated African trypanosomes is unknown. We showed that murine complement C3 fragments were deposited onto *T. congolense* when the trypanosomes were incubated with IgM anti-VSG and fresh mouse serum. We assessed whether complement receptor CR3 (CD11b/CD18) might be involved in mediating phagocytosis of *T. congolense*. We found that, in the presence of fresh mouse serum, there was significantly and markedly less phagocytosis of IgM-opsonized *T. congolense* by CD11b-deficient macrophages compared to phagocytosis by normal macrophages (78% fewer *T. congolense* were ingested per macrophage). There was significantly less TNF-α (38% less), but significantly more nitric oxide (NO) (63% more) released by CD11b-deficient

macrophages that had engulfed trypanosomes than by equally treated normal macrophages. We conclude that CR3 is a major, but not the only, receptor involved in IgM anti-VSG-mediated phagocytosis of *T. congolense* by macrophages. We further conclude that signaling via CR3, associated with IgM anti-VSG-mediated phagocytosis of *T. congolense*, either directly or indirectly enhance synthesis of disease-producing TNF-α, and inhibit synthesis of parasite-controlling NO.

3.2 Introduction

Trypanosoma congolense is a protozoan pathogen of cattle and other livestock. The parasite, like other members of the genus *Trypanosoma*, is responsible for the disease known as African trypanosomiasis (Mulligan & Potts, 1970). In the mammalian host, the whole parasite is coated with a glycoprotein coat of a single molecular species, called variant surface glycoprotein (VSG). African trypanosomes express about 10^7 densely packed identical VSG molecules on their surface (Cross, 1990). VSG is the major surface antigen and serves as a protective coat for the parasites.

The VSG is highly immunogenic and induces high levels of IgM antibodies in humans and animals infected with *T. brucei* or *T. congolense* (Binz et al., 1968, Clarkson, 1976, Greenwood & Whittle, 1973, Hudson et al., 1976, Luckins, 1976). IgM antibodies are the first and predominant class of anti-trypanosomal antibodies in mice and can be produced independently of T cell help (Campbell et al., 1978, Pinder et al., 1986). Antibodies to the VSG of *T. congolense* mediate control of parasitemia. In the absence of antibodies, bloodstream trypanosomes are protected against phagocytosis by the surface coat (Dempsey & Mansfield, 1983, Macaskill et al., 1980, Pinder et al.,

1986). Opsonization with VSG-specific antibodies results in rapid uptake of the parasites by Kupffer cells in the liver (Dempsey & Mansfield, 1983, Macaskill et al., 1980, Shi et al., 2004). IgM anti-VSG-mediated phagocytosis of *T. congolense* by macrophages has been shown by experiments carried out in vitro and in vivo (Kaushik et al., 1999, Shi et al., 2004). The macrophage receptor(s) recognizing IgM antibody-coated trypanosomes and enabling their phagocytosis is unknown.

IgM antibodies, after binding to antigen, are most efficient in activating the complement cascade (Law & Reid, 1988). It has been reported that phagocytosis of *T. congolense* (Shakibaei & Frevert, 1992) or *T. brucei* (Greenblatt et al., 1983, Macaskill et al., 1980) is optimal in the presence of functional complement. In African trypanosomiasis of humans, livestock and mice, the complement system is greatly activated, especially via the alternative pathway (Flemmings & Diggs, 1978, Malu & Tabel, 1986, Otesile et al., 1991).

The complement system is composed of some 30 proteins. Of all complement components, C3 is present at the highest concentration in plasma (~1.2 mg/ml) and holds a key position in the complement system (Law & Reid, 1988). Complement activation by immune complexes or antibody-opsonized cells results in the covalent attachment of C3b molecules to surface hydroxyl or amino groups. The plasma protease factor I, in synergy with factor H, rapidly cleaves surface-bound C3b into iC3b (May & Machesky, 2001). It is well established that the presence of iC3b on pathogens enhances their phagocytosis by macrophages and polymorphonuclear neutrophils by interacting with CR3 (Cain et al., 1987, Preynat-Seauve et al., 2004, Rosenthal et al., 1996, Ross & Medof, 1985). Therefore, it is conceivable that phagocytosis induced by IgM anti-VSG is mediated via CR3 on macrophages.

TNF-α is an inflammatory cytokine which exerts a wide range of biological activities including cellular proliferation and differentiation, apoptosis, cytotoxicity, inflammation and immunomodulation (Aggarwal & Natarajan, 1996). In African trypanosomiasis, TNF-α is a disease-enhancing cytokine produced by activated macrophages (Magez et al., 1999). Activated macrophages also secrete nitric oxide (NO) that is cytotoxic to *T. brucei* (Duleu et al., 2004, Gobert et al., 1998, Vincendeau & Daulouede, 1991) and *T. congolense* (Kaushik et al., 1999).

The purpose of this study was to define whether CR3 (CD11b/CD18) is one of the receptors involved in IgM-mediated phagocytosis of *T. congolense* by macrophages and to investigate potential signaling via CR3.

We exposed normal and CD11b-deficient murine peritoneal macrophages to *T. congolense* together with IgM anti-VSG antibody and fresh mouse serum and compared the degree of phagocytosis by immunocytochemistry. We show that, in the presence of fresh mouse serum, there is significantly less phagocytosis of IgM-opsonized *T. congolense* by CD11b-deficient macrophages compared to that by normal macrophages. In addition, our results show that there is significantly less TNF-α, but significantly more NO, released by CD11b-deficient macrophages that have engulfed trypanosomes than by equally treated normal macrophages. Together these results suggest that CR3 is the major, but not the only, receptor on macrophages involved in IgM anti-VSG-mediated phagocytosis of *T. congolense*. The biological significance of the subsequently enhanced synthesis of TNF-α and decreased NO production by CD11b-deficient macrophages will be discussed.

We also investigated a potential correlation between deposition of C3b onto target cells, via activation of the alternative complement pathway, and the survival of mice infected with *T. congolense*.

The alternative pathway of complement activation in susceptible BALB/c differs from that of relatively resistant C57BL/6 mice (Ogunremi et al., 1993). We asked the question whether the differences in the alternative pathways of complement activation might correlate with resistance to *T. congolense* infections. By cross-breeding BALB/c and C57BL/6 mice, we generated a population of F2 offspring. We then investigated whether the deposition of plasma C3b/iC3b (CR3 ligand), via activation of the alternative complement pathway, onto zymosan correlated with the survival of these mice infected with *T. congolense*.

3.3 Materials and Methods:

Parasites

T. congolense, Trans Mara strain, variant antigenic type (VAT) TC13 was used in this study. The origin of this parasite strain has been previously described (Tabel, 1982). Frozen stabilates of parasites were used for infecting CD1 mice immunosuppressed with cyclophosphamide, and passages were made every third day as described previously (Tabel, 1982). The parasites purified from the blood of infected CD1 mice by DEAE-cellulose chromatography (Lanham & Godfrey, 1970) were used for co-culture with macrophages.

Antibodies

The rat hybridoma MCAP497 (IgG2a isotype) (specific for mouse macrophage antigen F4/80) was purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, U.S.A). Biotin-conjugated goat anti-rat IgG2a was purchased from Cedarlane (Hornby, Ontario, Canada). Biotin-conjugated goat anti-rabbit IgG was purchased from Vector Laboratories (Burlingame, CA). Goat anti-rabbit IgG conjugated with Alexa Fluor 546 and streptavidin Alexa Fluor 488 were obtained from Molecular Probes (Eugene, Oregon, USA). Recombinant TNF-α and antibodies against TNF-α were purchased from BD Biosciences PharMingen (San Diego, CA). Recombinant IFN-γ was purchased from R&D Systems Inc. (Minneapolis, USA). Production of monoclonal anti-mouse complement component C3 11H9 has been previously described (Kremmer et al., 1990). Peroxidase-labelled goat anti-mouse C3 was obtained from Cappel Laboratories (Cochranville, Pa., USA). The production of the polyclonal rabbit anti-*T. congolense* (Shi et al., 2003), and of monoclonal antibodies (mAb) 6C1 and 5A8 (IgM) specific for the VSG of VAT TC13 has been described (Wei et al., 1990).

Mice

Eight to 10 week-old, female, CD11b-deficient mice were obtained from Jackson Laboratories (genetic background: C57BL/6; strain name: B6.129S4-Itgam^{tm1Myd}/J; stock number: 003991). Eight to 10 week-old C57BL/6 and BALB/c mice of both sexes and 5 to 8 week-old, female, Swiss white mice (CD1) were purchased from the Animal Resource Center of the University of Saskatchewan (Saskatoon, Canada). The mice were kept in polycarbonate cages on sawdust, and allowed free access to food and water

throughout the experiments, according to the recommendations of the Canadian Council of Animal Care.

Breeding of mice

BALB/c and C57BL/6 mice were cross-bred to produce F2 progeny as described (Ogunremi & Tabel, 1995). Female offspring were identified at weaning and were bled for plasma at 8½ weeks of age. At 10 weeks, they were infected with *T. congolense.*

Mouse plasma and serum

Fresh mouse serum was collected from female 5 to 8 week-old CD1 mice. Heat-inactivated mouse serum was obtained by incubating fresh CD1 mouse serum at 56°C for 30 min. Plasma samples were collected from 8 to 10-week-old female BALB/c and C57BL/6 mice or 8½ week-old F2 mice into EDTA microtainer tubes (Becton Dickinson, Rutherford, N. J., USA).

Preparation of peritoneal macrophages

Peritoneal macrophages were isolated from 8 to 10-week-old (C57BL/6 and CD11b-deficient C57BL/6) mice 3 days following i.p. injection of 0.3 ml pristane (Sigma, Oakville, Ontario, Canada). Mice were euthanized by CO_2. Peritoneal lavage was performed with 10 ml DMEM complete medium (DMEM medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, and 100 IU of penicillin and streptomycin [Life Technologies, Grand Island, NY]). Collected peritoneal cells were washed twice in DMEM complete medium, and resuspended in DMEM complete medium. Peritoneal cells were allowed to adhere

to tissue culture plastic for 2 h at 37^0C, after which non-adhered cells were removed by rinsing the cell monolayer with medium (Shakibaei & Frevert, 1992).

Test for covalent binding of C3 fragments onto T. congolense

Plasma from two mouse strains (BALB/c and C57BL/6) was used in the assay. $MgCl_2$ and EGTA (30 mM and 5 mM respectively) (Sigma, Oakville, Ontario, Canada) were added to plasma (Mg-EGTA plasma). *T. congolense* organisms (10^7) in 20 µl tris-buffer-saline containing 1.25% glucose and 0.1% gelatin were added to 100 µl Mg-EGTA plasma. Then, 2 µl of mAb 5A8 (IgM anti-VSG) was added to the plasma-trypanosome mixture to give a final antibody to plasma dilution of 1:50. This dilution had previously been determined to be optimal for C3 deposition on trypanosomes. Incubation was done at 37^0C for 20 min, after which the reaction was stopped by placing the vial on ice. The mixture was spun at 1000 × g for 10 min, the supernatant aspirated, and the trypanosomes washed thrice with Tris-buffer-saline containing glucose and gelatin. After the final wash, trypanosomes were treated with 100 µl of 100 mM methylamine (Sigma) to elute covalently bound C3 fragments (Nilsson et al., 1989, Ogunremi et al., 1993). Controls included reactions of trypanosomes with Mg-EGTA plasma only (i.e. no anti-*T. congolense* mAb), as well as trypanosomes alone (i. e. no Mg-EGTA plasma). The C3 fragments present in the eluate were quantified using the C3 sandwich ELISA as described (Ogunremi et al., 1993). ELISA results are expressed as C3 units, which were calculated from a standard curve of the OD_{405} readings of plasma C3 plotted against the log of dilutions of normal plasma ($p< 0.01$).

Phagocytosis of T. congolense in vitro

Peritoneal macrophages were seeded in Lab-Tek 16-chamber glass slides (Electron Microscopy Sciences, Washington, USA) at 5×10^4 per well in 200 µl DMEM complete medium with 12 ng/ml IFN-γ (R&D systems, Minneapolis, USA) for 24 hr at 37^0C. Then, the cells were washed 2x with DMEM. The macrophages were co-cultured with freshly isolated *T. congolense* (macrophages: trypanosome ratio = 1:10) in DMEM complete medium for 1 hr at 37^0 C. The medium in the phagocytosis experiments contained 50% fresh mouse serum instead of 10% heat-inactivated fetal bovine serum. MAb 6C1 (IgM anti-VSG) was added to cultures at various concentrations. After incubation, the chambers were rinsed with PBS, the plastic case removed, and the cells on the microscope slide were stained with fluorescent antibodies. The percentage of the numbers of macrophages that had engulfed trypanosomes was determined by examining 200 cells at random. The numbers of *T. congolense* ingested per macrophage were determined by using Northern Eclipse software from Empix Imaging Inc. (Mississauga, ON, Canada). The results for each experiment were obtained by examining 200 cells at random. In a preliminary experiment, the time course of phagocytosis (10, 20, 30, 60 min) had been established. The phagocytosis reached the maximum at 60 min incubation of macrophages with *T. congolense* and IgM anti-VSG antibody (data not shown). The 60 min incubation was used as a standard for the following experiments. In the presence of fresh mouse serum, phagocytosis of *T. congolense* by macrophages was assessed at antibody concentrations of 1.25 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml and 25 µg/ml. Phagocytosis of *T. congolense* by macrophages was found to occur in an antibody dose-dependent pattern (data not shown). At the antibody concentration of 25 µg/ml, almost

100% of cells showed phagocytosis (data not shown). We used a sub-optimal antibody concentration (10 μg/ml) for the experiments described below.

Immunocytochemistry

Immunofluorescent double staining of macrophages that had engulfed trypanosomes has been described (Shi et al., 2004).

TNF-α assays

Peritoneal macrophages were cultured in 24-well plates at the concentration of 6×10^5 cells/ml/well. Cells grown in DMEM complete medium contained 50% fresh mouse serum, instead of 10% heat-inactivated fetal bovine serum, served as negative control. The principal cultures contained macrophages plus *T. congolense* and mAb 6C1 as described above (phagocytosis of *T. congolense* in vitro). The cell culture supernatant fluids were harvested after 4 hr incubation. Culture supernatant fluids were centrifuged for 5 min at 1000 x g to remove cellular debris, transferred to new tubes, and stored at -80^0 C until analysis. The levels of TNF-α in the culture supernatant fluids were determined by routine sandwich ELISA by using immulon-4 plates and TNF-α ELISA set (BD Biosciences Pharmingen, San Diego, CA), according to the manufacturer's protocol. Each sample of cell culture fluid was tested for TNF-α in triplicate. The detection limit of the ELISA assays was 31 pg/ml.

Measurement of nitrite production

Nitrite concentrations in the culture fluids harvested after 24 hr were determined by the "Griess reagent system" from Promega (Madison, WI, U.S.A.) as described previously (Kaushik et al., 1999). Briefly, 50 μl of culture supernatant fluids were incubated with an equal volume of sulfanilamide solution (1% sulfanilamide [Sigma] in 5% phosphoric acid [Sigma]) for 5-10 min at room temperature, protected from light. Then 50 μl of the NED (Sigma) (0.1% N-1-napthylethylenediamine dihydrochloride in water) was added and incubated for another 10 min at room temperature, protected from light. The absorbance was measured at 550 nm in a micro-ELISA reader. Nitrite levels were determined by comparison with a sodium nitrite (Sigma) standard curve. The sensitivity of this assay was 2.5 μM.

Generation and detection of zymosan-bound complement C3 fragments

The methods have been described previously (Ogunremi et al., 1993). Briefly, 100 μl of mouse plasma containing 30 mM Mg^{2+} and 5 mM EGTA was incubated with 1 mg zymosan (Sigma) for 10 min. Ca^{2+}, which normally serves as a ligand in the trimolecular complex C1qrs of the classical pathway (Bryant & Jenkins, 1968), is not involved in the alternate pathway of complement activation (Fine, 1977). In contrast, Mg^{2+} is required for C3 convertase formation by both classical and alternate pathways (Muller-Eberhard et al., 1967, Pangburn et al., 1981). EGTA has a high affinity for Ca^{2+} but has a much lower association constant for Mg^{2+}, and therefore allows activation by the alternate but not the classical pathway (Des Prez et al., 1975, Fine, 1977). The addition of Mg^{2+} to form MgEGTA allows optimum activation of the complement

alternative pathway (Des Prez et al., 1975, Fine, 1977). The zymosan was thoroughly washed and the C3 fragments eluted by treatment with 100 mM methylamine (Sigma) for 30 min at room temperature. The concentrations of eluted C3 fragments were determined by ELISA.

Statistical analysis

Data are presented as means ± standard error (SE). Analysis of Variance (ANOVA) and regression analysis were carried out using Excel software (Microsoft, Santa Monica, CA, USA). A P value < 0.05 was considered statistically significant.

3.4 Results

IgM anti-VSG mediates covalent binding of C3 fragments onto T. congolense

Antibody-mediated lysis of *T. brucei rhodesiense* (Flemmings & Diggs, 1978) and *T. congolense* (Liu et al., 1993) occurs via activation of the alternative pathway of complement. We investigated whether IgM anti-VSG induces deposition of murine C3 fragments onto *T. congolense* under conditions that favor activation of the alternative complement pathway. Live *T. congolense* were incubated with mouse plasma samples (containing 5 mM EGTA and 30 mM $MgCl_2$) from C57BL/6 or BALB/c mice in the presence of IgM anti-VSG antibody. Murine C3 fragments were detectable on trypanosomes when the parasites were incubated with, but not without specific antibody (Fig 3.1). More C3 fragments were deposited onto the trypanosomes when the parasites were incubated with C57BL/6 plasma rather than with plasma derived from BALB/c mice ($p< 0.05$). These data indicate that IgM anti-VSG antibodies induce C3 fragments

to covalently bind onto *T. congolense*. They also show that the complement cascade of the relatively resistant C57Bl/6 mice allowed deposition of significantly more C3 fragments onto *T. congolense* than the complement cascade of the susceptible BALB/c.

FIGURE 3.1

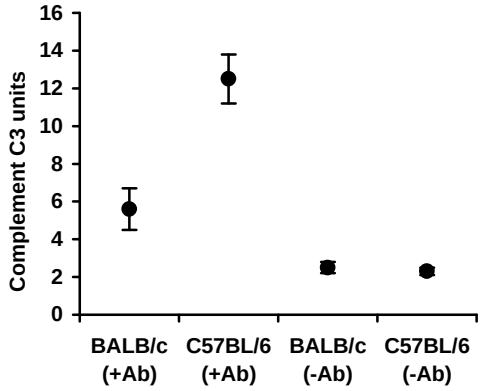


FIGURE 3.1* IgM anti-VSG mediates covalent binding of C3 fragments onto *T. congolense*. *T. congolense* were incubated with EGTA/Mg^{++} plasma from C57BL/6 and BALB/c mice in the presence (+Ab) or absence (-Ab) of monoclonal IgM anti-VSG. The trypanosome-bound C3 fragments were eluted with 0.1 M methylamine. Eluted C3 fragments were quantified by a sandwich ELISA consisting of a monoclonal anti-mouse C3 mAb 11H9 and a peroxidase-labelled goat anti-mouse C3 antiserum. Error bars show standard deviation from the mean of four separate experiments.

*** Data for Figure 3.1 were produced by Dele Ogunremi, and reported on page 84 in his PhD. Thesis (1993).**

CR3 is the major macrophage receptor involved in IgM anti-VSG-mediated phagocytosis of T. congolense

To assess whether CR3 is involved in IgM anti-VSG mediated phagocytosis of *T. congolense*, peritoneal macrophages from CD11b-deficient and normal mice were examined for phagocytosis of *T. congolense* in the presence of IgM anti-VSG antibody and fresh mouse serum. *T. congolense* was detected with the use of rabbit anti-*T. congolense* antiserum (red) and macrophages with the use of anti-F4/80 (green) by immunocytochemistry (Fig. 3.2). The intracellular localization of *T. congolense* ingested by macrophages was confirmed by comparing the immunocytochemistry results with and without permeabilization of cells by saponin (Fig. 3.2). There were significantly fewer (78%) *T. congolense* ingested per CD11b-deficient macrophage than per normal macrophage (1.31±0.23 vs. 5.88±1.20) (Fig. 3.3A). From the distribution data of the numbers of *T. congolense* ingested per macrophage, we observed that more than 50% of CD11b-deficient macrophages that did show phagocytosis ingested less than 2 parasites per cell. However, in more than 50% of normal macrophages that did show phagocytosis, 2 to 8 parasites were ingested per cell (Fig. 3.3B). There were also significantly fewer (43%) CD11b-deficient macrophages than normal macrophages that showed phagocytosis of *T. congolense* (51.0±1.0% vs. 89.1±3.2%) (Fig. 3.4). These data indicate that CR3 is the major receptor involved in IgM anti-VSG-mediated phagocytosis of *T. congolense* by macrophages.

Peritoneal macrophages from normal and CD11b-deficient mice were examined for phagocytosis of complement-opsonized *T. congolense* (incubated with fresh mouse serum) versus non-complement-opsonized *T. congolense* (incubated with heat-inactivated mouse serum) in the presence of IgM anti-VSG antibody. Nearly equivalent

degrees of phagocytosis of *T. congolense* were observed under the following three conditions: (1) CD11b-deficient macrophages with fresh mouse serum and IgM anti-VSG; (2) CD11b-deficient macrophages with heat-inactivated mouse serum and IgM anti-VSG; (3) normal mouse macrophages with heat-inactivated mouse serum and IgM anti-VSG. The numbers of *T. congolense* ingested per macrophage were 1.31±0.23, 1.40±0.14 and 1.50±0.20, respectively (Fig. 3.3A). The proportions of macrophages that showed phagocytosis were 51±1.0%, 50.6±5.9% and 52.7±3.2%, respectively (Fig. 3.4). These data indicate that IgM-anti-VSG mediated phagocytosis via CR3 is a complement-dependent process.

Peritoneal macrophages from CD11b-deficient and normal mice were also examined for up-take of *T. congolense* in the presence of fresh mouse serum but absence of IgM anti-VSG antibody. Immunocytochemistry results showed that, without IgM anti-VSG antibody, only a negligible number of *T. congolense* was ingested (Fig. 3.3A, 3.4) by both kinds of macrophages. These data indicate that anti-VSG antibodies are necessary for effective phagocytosis of *T. congolense* by macrophages. This finding confirms our previous *in vitro* and *in vivo* results (Kaushik et al., 1999, Shi et al., 2004).

FIGURE 3.2

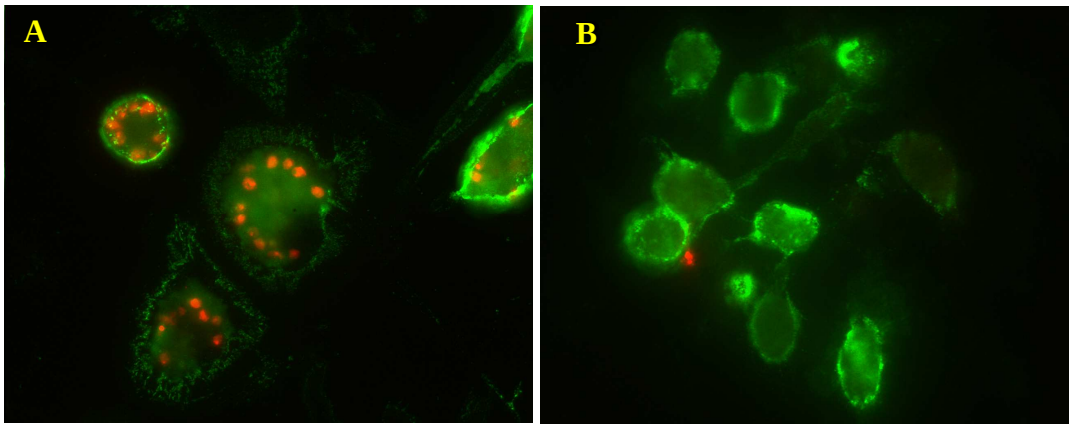


FIGURE 3.2 IgM anti-VSG-mediated phagocytosis of *T. congolense* by murine peritoneal macrophages in the presence of fresh mouse serum. Normal peritoneal macrophages were incubated with *T. congolense* and IgM anti-VSG in the presence of fresh mouse serum. *T. congolense* was detected with the use of rabbit anti-*T. congolense* antiserum (red). Macrophage was detected with the use of anti-F4/80 (green) (1000× magnification). Cells were stained in the presence (A) or absence (B) of saponin that mediates permeabilization of the cell membrane.

FIGURE 3.3

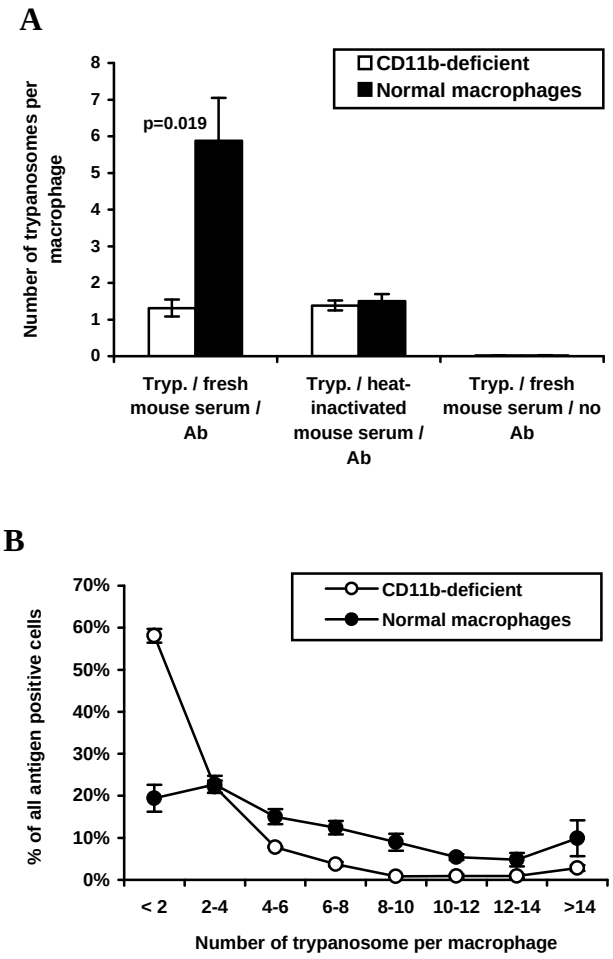


FIGURE 3.3 Phagocytosis of *T. congolense* by CD11b-deficient and normal macrophages: A, Numbers of *T. congolense* ingested per macrophage. **B, Range of distribution of numbers of *T. congolense* ingested per macrophage.** Peritoneal macrophages from CD11b-deficient mice or normal mice were incubated with *T. congolense* in the presence or absence of IgM anti-VSG and fresh mouse serum for 1hr at 37^0C. *T. congolense* was detected with the use of rabbit anti-*T. congolense* antiserum (red). Macrophage was detected with the use of anti-F4/80 (green). *T. congolense* ingestion per macrophages was determined by measuring the red area (*T. congolense* antigen) in the green cell (macrophage) using Northern Eclipse software. In each experiment, 200 cells were examined at random. The data shown are the means of three separate experiments.

FIGURE 3.4

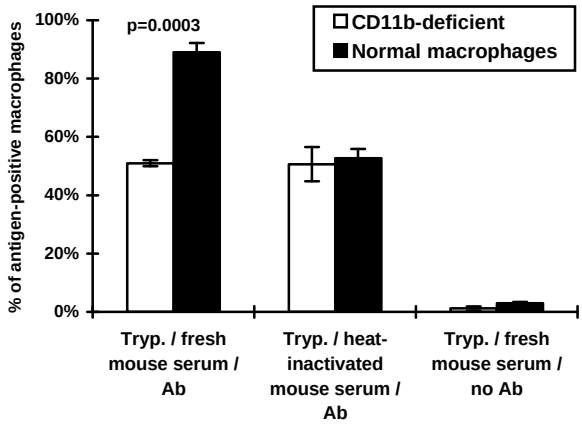


FIGURE 3.4 Phagocytosis of *T. congolense* by CD11b-deficient and normal macrophages: Numbers of *T. congolense* antigen-positive cells. Peritoneal macrophages from CD11b-deficient mice and normal mice were incubated with *T. congolense* in the presence or absence of IgM anti-VSG and fresh mouse serum for 1hr at 37^0C. *T. congolense* was detected with the use of rabbit anti-*T. congolense* antiserum (red). Macrophage was detected with the use of anti-F4/80 (green). The percent of macrophages that had engulfed trypanosomes was determined by examining 200 cells at random. The data shown are the means of three separate experiments.

Signaling via CR3, associated with IgM anti-VSG-mediated phagocytosis of* T. congolense*, affects synthesis of TNF-α

We next investigated the role of CR3 in the synthesis of TNF-α. Peritoneal macrophages from CD11b-deficient and normal mice were stimulated with or without IFN-γ (12 ng/ml) for 24 hr, and then incubated with *T. congolense* with fresh mouse serum in the presence or absence of IgM anti-VSG. There was significantly less TNF-α (38% less) released by CD11b-deficient macrophages that had engulfed trypanosomes than by equally treated normal macrophages (991±69.4 pg/ml vs. 1594±29.7 pg/ml, respectively) (Fig. 3.5). The data indicate that synthesis of TNF-α, associated with phagocytosis of *T. congolense*, is influenced by CR3 signaling either directly or indirectly.

FIGURE 3.5

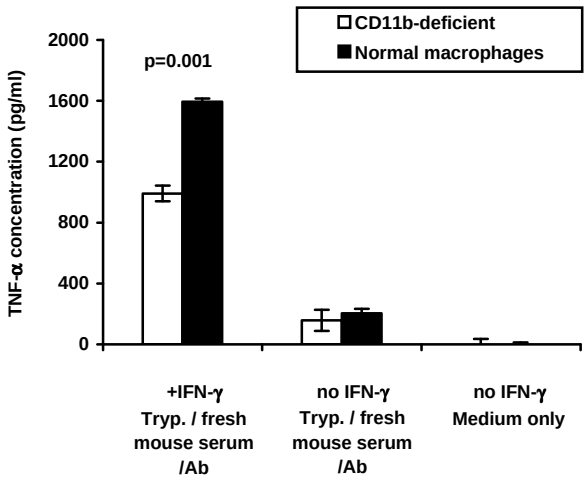


FIGURE 3.5 Signaling via CR3, associated with IgM anti-VSG-mediated phagocytosis of *T. congolense,* affects synthesis of TNF-α. Peritoneal macrophages (6×10^5 cells per well) were seeded in 24-well plates in 1 ml DMEM complete medium with or without 12 ng IFN-γ for 24hr at 37^0C. Then the cells were cultured in DMEM with *T. congolense* (macrophage : trypanosome ratio = 1:10) in the presence of 50% fresh mouse serum and IgM anti-VSG mAb 6C1 (10 μg/ml). Negative control cultures consisted of cell cultures to which only DMEM and 50% fresh mouse serum was added. The cell culture supernatants were harvested after 4hr incubation. The TNF-α concentrations of the supernatants were determined by routine sandwich ELISA. The data shown are from one experiment and are representative of four separate experiments.

Signaling via CR3, associated with IgM anti-VSG-mediated phagocytosis of T. congolense, inhibits synthesis of nitric oxide (NO)

Macrophage-derived NO has been found to have a cytotoxic effect on *T. brucei* (Duleu et al., 2004) as well as on *T. congolense* (Kaushik et al., 1999). Peritoneal macrophages from CD11b-deficient and normal mice were stimulated with or without IFN-γ (12 ng/ml) for 24 hr, and then incubated with *T. congolense* in the presence of IgM anti-VSG and fresh mouse serum. Both CD11b-deficient and normal macrophages produced much less NO upon IgM anti-VSG-mediated phagocytosis when previously cultured without IFN-γ stimulation (Fig. 3.6). Although there were less *T. congolense* ingested, there was significantly more NO (63% more) released by CD11b-deficient macrophages that had engulfed trypanosomes than by equally treated normal macrophages (10.72±1.05 μM vs. 3.95±0.30 μM respectively) (Fig. 3.6). These data indicate that signaling via CR3, associated with IgM anti-VSG-mediated phagocytosis of *T. congolense*, either directly or indirectly inhibits NO synthesis.

FIGURE 3.6

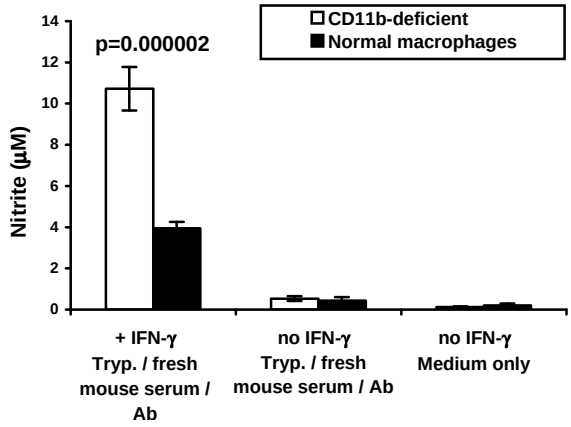


FIGURE 3.6 Signaling via CR3, associated with IgM anti-VSG-mediated phagocytosis of *T. congolense*, inhibits synthesis of nitric oxide. Peritoneal macrophages (6×10^5 cells per well) were seeded in 24-well plates at in 1 ml DMEM complete medium with or without 12 ng IFN-γ for 24 hr at 37^0C. Then, the cells were cultured in DMEM with *T. congolense* (macrophage: trypanosome ratio = 1:10) in the presence of 50% fresh mouse serum and mAb 6C1 (IgM anti-VSG) at a concentration of 10 μg/ml. Negative control macrophage cultures contained neither trypanosomes nor antibodies. The cell culture supernatants were harvested after 24 hr incubation. The nitrite concentrations of the supernatants were determined by the Griess reagent. The data shown are the means of four separate experiments.

Negative correlation between deposition of C3 fragments onto zymosan via the alternative pathway of complement and the survival period in T. congolense-infected progenies of BALB/c x C57BL/c crosses

Since we found divergent effects of CR3 signaling for the synthesis of TNF-α and NO, we turned our attention to the ligand of CR3, i.e. iC3b, the degradation product of C3b.

We previously observed differences in the activity of the alternative pathway of complement in BALB/c and C57BL/6 mice. Deposition of more C3b occurred when zymosan was incubated with C57BL/6 plasma (Ogunremi et al., 1993). BALB/c mice are highly susceptible to *T. congolense* infection whereas C57BL/6 mice are relatively resistant (Tabel et al., 2000). We also found more C3 fragments being deposited onto *T. congolense* when the parasite was incubated with plasma of C57BL/6 mice (Fig. 3.1). We asked the question whether deposition of different amounts of C3b onto target cells, via activation of the alternative complement pathway, might correlate with the genetic differences in resistance of mice to *T. congolense* infection. We infected 93 F2 mice from both reciprocal BALB/c x C57BL/6 crosses with 10^3 *T. congolense*. Prior to infection, we bled these mice and subjected each plasma sample to zymosan opsonization assay for determination of the amount of deposited C3b. We then assessed the correlation of amounts of deposited C3b and survival times of the infected mice. Rather than finding a positive correlation, which we had anticipated, we found a low, but statistically significant negative correlation ($r=-0.32$; $p=0.002$) (Fig. 3.7). Considering these observations, we suggest that deposition of C3b onto trypanosomes might not enhance but slightly decrease the chance of the host to resist *T. congolense* infection. Further, we suggest that the relatively resistant C57BL/6 mice might carry a

susceptibility gene coding for some, yet unknown protein of the alternative complement cascade.

FIGURE 3.7

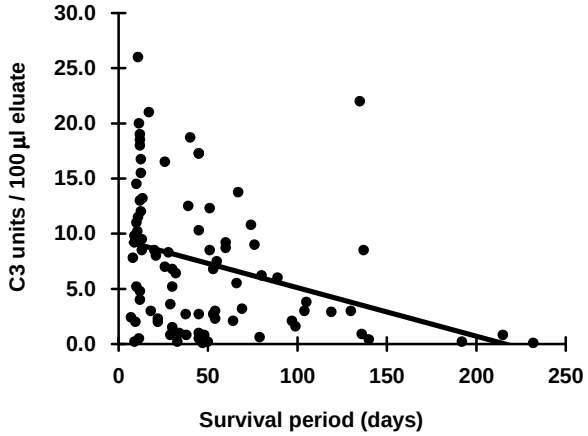


FIGURE 3.7* Relationship between deposition of complement C3 fragments onto zymosan and resistance to *Trypanosoma congolense* infection in F2 progeny of BALB/c x C57BL/6 crosses. Correlation was assessed by regression analysis: r = -0.32, p = 0.002. Each mouse was pre-bled for plasma (for zymosan opsonization assay) before infection. Results of the F2 mice from both reciprocal BALB/c x C57BL/6 crosses are presented (n = 93). Each mouse was infected with 10^3 *T. congolense* and monitored for survival period as an indication of resistance. Each data point represents one mouse.

*** Data for Figure3.7 were produced by Dele Ogunremi, and reported on page 93 in his PhD. Thesis (1993).**

3.5 Discussion

In the host defense system, macrophages play an important role through their ability to remove specific substances from the blood via various receptors, such as complement receptors, Fc-receptors, scavenger receptors and mannose receptors (Aderem & Underhill, 1999, Linehan et al., 2000). The control of parasitemia in African trypanosomiasis is mediated by at least two known mechanisms: (1) antibody/complement-mediated lysis and (2) antibody-mediated phagocytosis (Tabel et al., 2000). A possible third mechanism, i. e. release of trypanotoxic NO by macrophages after antibody-mediated phagocytosis remains controversial (Duleu et al., 2004, Gobert et al., 1998, Kaushik et al., 1999, Sternberg et al., 1994, Vincendeau & Daulouede, 1991). IgM antibody plays an important role in the initial stages of immunity. IgM antibodies are the first predominant class of anti-trypanosomal antibodies in infections with African trypanosomes (Binz et al., 1968, Clarkson, 1976, Greenwood & Whittle, 1973, Hudson et al., 1976, Luckins, 1976). IgM antibodies, after binding to antigen, are most efficient in activating the complement cascade (Law & Reid, 1988) and trigger abundant deposition of C3b. Our data show that C3 fragments covalently bind onto the parasites in the presence of IgM anti-VSG (Fig. 3.1). C3b covalently bound to target cells can quickly be degraded to iC3b by plasma factor I. In blood, C3b has a much shorter half life than iC3b (90 s vs. 45 min) (Cain et al., 1987, Ross, 1986). CR3, the receptor for iC3b, has been shown to be involved in phagocytosis of IgM-opsonized erythrocytes by murine Kupffer cells (Yan et al., 2000). We have shown that IgM anti-VSG-opsonized *T. congolense* are phagocytosed by Kupffer cells in the liver (Shi et al., 2004).

In the present work, we observed an important role for CR3 in IgM anti-VSG-mediated phagocytosis of *T. congolense* by macrophages. We found that ingestion of IgM anti-VSG opsonized *T. congolense* by CD11b-deficient macrophages was consistently lower than that by normal macrophages, both by measuring the numbers of *T. congolense* ingested per macrophage (Fig. 3.3A, B) as well as the percentage of the numbers of macrophages that showed phagocytosis (Fig. 3.4). This indicated that CR3 is the main receptor involved in IgM anti-VSG-mediated phagocytosis of *T. congolense*, but is not the only receptor involved. The involvement of CR3 in phagocytosis of antibody-coated targets other than trypanosomes has been documented (Aderem & Underhill, 1999, Law & Reid, 1988, Yan et al., 2000). The existence of Fc receptors for IgM on murine and human T, B and NK cells as well as macrophages has been reported (Shibuya et al., 2000). Their biological significance in phagocytosis of pathogens remains to be determined.

Several studies have reported that CR3, in addition to its role in complement-opsonized phagocytosis, serves in the nonopsonic recognition of microbes by interacting directly with certain microbial molecules on their surfaces (Le Cabec et al., 2002, Yan et al., 2000). In the current study, we found that the IgM anti-VSG-mediated phagocytosis of *T. congolense* via CR3 is entirely complement-dependent. Equivalent degrees of phagocytosis of IgM anti-VSG-opsonized *T. congolense* by CD11b-deficient macrophages were observed, no matter whether functional complement was present or absent (Fig. 3.3A and 3.4). The ligand for CR3 is most likely iC3b, which is covalently bound to *T. congolense* by the activation of complement system via antibody-antigen complex (Fig. 3.1). The iC3b-coated parasites are then engulfed by macrophages via CR3. The localization of the relevant iC3b on *T. congolense* remains to be determined.

iC3b binds to the surface carbohydrate of antigens (Law & Reid, 1988). Since the C3-fragments covalently bound to *T. congolense* could be eluted by methylamine (Fig. 3.1), it is safe to assume that they were bound to trypanosomal carbohydrate. We speculate that they were bound to trypanosomal GIP.

Since CR3 deficiency was not associated with a complete lack of IgM anti-VSG-mediated phagocytosis of *T. congolense*, other receptors must be involved in this process. The relevant receptors are presently unknown. We consider the mannose receptor a possible candidate (Linehan et al., 2000). Anti-VSG antibodies induce *T. congolense* to shed VSG (Liu et al., 1993) (see below). This process might expose invariant surface epitopes, such as the conserved VSG carbohydrates (Gerold et al., 1996, Magez et al., 2002). Mannose receptor of macrophages might recognize these carbohydrate epitopes and further mediate phagocytosis.

We also demonstrated that IgM anti-VSG-mediated phagocytosis of *T. congolense* via CR3 receptor by macrophages is associated with signaling of TNF-α synthesis. Studies have shown that deposition of complement component onto the surface of Group B *Streptococcus* (GBS) and subsequent CD11b-mediated activation is the central mechanism by which plasma or serum potentiates GBS-induced TNF-α release from human monocytes in culture and in whole blood (Levy et al., 2003). Bacterial internalization via CR3 (CD11b/CD18) accounts for a substantial fraction of the induction of cytokine (TNF-α) signaling (Moore et al., 2000). We observed that significantly less TNF-α was released by CD11b-deficient macrophages that had engulfed trypanosomes than by equally treated normal macrophages (Fig. 3.5). These data indicate that CR3-signaling, associated with IgM anti-VSG-mediated phagocytosis

of *T. congolense*, either directly or indirectly enhances synthesis of TNF-α. However, other, yet unknown receptors must be involved. TLR2 is a potential candidate. For example, stimulation of TLR2 by glycosylphosphatidylinositol from *Trypanosma cruzi* was found to induce synthesis of TNF-α in macrophages (Campos et al., 2001).

Induction of tumor necrosis factor (TNF-α) and its role in immunopathology has been extensively documented in African trypanosomiasis (Hunter et al., 1991, Magez et al., 1999, Magez et al., 2002, Okomo-Assoumou et al., 1995, Sileghem et al., 1994b). Enhanced expression of TNF-α in the brains of *Trypanosoma brucei*-infected mice has been reported (Hunter et al., 1991). Associations have been found between TNF-α production by monocytes and the severity of disease-associated anemia in trypanosome-infected cattle (Sileghem et al., 1994b), neuropathological symptoms in human sleeping sickness patients (Okomo-Assoumou et al., 1995), trypansosome-elicited immunosuppression and overall morbidity (Magez et al., 1999). It is tempting to speculate that CR3-mediated phagocytosis of *T. congolense* in the presence of IgM antibody might contribute to enhanced trypanosomiasis-associated immunopathology by increasing synthesis of TNF-α.

On the other hand, the results presented in this study support the conclusion that signaling via CR3, associated with IgM antibody-mediated phagocytosis of *T. congolense*, either directly or indirectly inhibit the synthesis of NO. Our data showed that CD11b-deficient macrophages that had engulfed fewer trypanosomes produced significantly more NO than equally treated normal macrophages (Fig. 3.6). Activated macrophages express inducible NO synthase (iNOS) which synthesizes large amounts of NO by oxidation of L-arginine (Munder et al., 1998). It has been reported that *T. brucei*

brucei directly activates arginase, another enzyme that metabolizes L-arginine. When the arginase pathway is activated, it inhibits NO production (Duleu et al., 2004). The observed enhanced NO production in CD11b-deficient mice could be either due to a reduced inhibitory signal by CR3 or due to a reduced inhibitory, intracellular signal because of less intracellular trypanosomes. Presently it is unknown which mechanism might have been operating. Our previous studies on phagocytosis of *T. congolense* by bone marrow-derived macrophages from both C57BL/6 and BALB/c mice showed that phagocytosis of *T. congolense* mediated by anti-VSG antibodies of IgG2a isotype induced two- to nine-fold more NO than phagocytosis mediated by IgM antibodies (Kaushik et al., 1999). Increase of IgM anti-VSG correlated with decreased NO production (Kaushik et al., 1999). It has been observed that TLR5/TLR4 complexes are involved in NO synthesis signaling of macrophages stimulated with bacterial flagellin (Mizel et al., 2003). Macrophage mannose receptors may also be involved in NO synthesis signaling (Karaca et al., 1995).

NO has been shown to be trypanostatic for *T. congolense*, *T. musculi*, and *T. brucei in vitro* (Duleu et al., 2004, Gobert et al., 1998, Kaushik et al., 1999, Vincendeau & Daulouede, 1991, Vincendeau et al., 1992). Peritoneal macrophages and bone marrow-derived macrophages from resistant C57BL/6 mice produce significantly higher NO than macrophages of susceptible BALB/c mice in response to *T. congolense* (Kaushik et al., 1999). Therefore, NO may at least partially account for differences in resistance to *T. congolense* infection. Thus, we speculate that stimulation of CR3 during IgM anti-VSG-mediated phagocytosis of *T. congolense* impairs control of parasitemia by inhibiting NO production, and thus reducing the cytostatic effect on *T. congolense*. If this hypothesis proves to be correct, it might explain why enhanced deposition of C3b

onto target cells correlates with susceptibility rather than resistance to *T. congolense* infection (Fig. 3.7).

CR3 contributes to both beneficial and detrimental effects for controlling the disease. The beneficial effect is that CR3 is one of the receptors involved in antibody-mediated phagocytosis, which is a major immune response mediating control of parasitaemia (Dempsey & Mansfield, 1983, Macaskill et al., 1980, Pinder et al., 1986, Shi et al., 2003). Stimulation of CR3 during IgM anti-VSG-mediated phagocytosis increases synthesis of TNF-α that enhances trypanosomiasis-associated immunopathology (Hunter et al., 1991, Magez et al., 1999, Okomo-Assoumou et al., 1995, Sileghem et al., 1994b) and inhibits synthesis of NO that is involved in cytotoxicity to *T. brucei* and *T. congolense* (Duleu et al., 2004, Gobert et al., 1998, Kaushik et al., 1999, Vincendeau & Daulouede, 1991).

In conclusion, the results presented in this report indicate that CR3 is the major receptor involved in IgM anti-VSG mediated phagocytosis of *T. congolense*. We suggest that signaling via CR3 during IgM anti-VSG-mediated phagocytosis of trypanosomes enhance disease by increased synthesis of TNF-α and by decreased NO-mediated control of parasitemia.

4. RELESAE OF SOLUBLE VARIANT SURFACE GLYCOPROTEIN FROM *TRYPANOSOMA CONGOLENSE*: EFFECT OF ANTIBODY AND COMPLEMENT

4.1 Abstract

The surfaces of African trypanosomes are covered with a layer of a single species of glycoprotein, called variant surface glycoprotein (VSG). Soluble VSG (sVSG) has been reported to induce polyclonal B cell activation, to induce macrophages to produce TNF-α and inhibit macrophages to synthesise NO. In this study, we assessed the effect of IgM anti-VSG antibody on the release of sVSG from *T. congolense.* We found that there was more sVSG released from *T. congolense* by interaction with IgM anti-VSG than by interaction with equal amounts of IgG2a anti-VSG. The release of sVSG occurred in an antibody dose-dependent pattern. We also found that IgM anti-VSG forms soluble immune complexes with sVSG. The results also showed that antibody-induced release of sVSG can occur without complement, but is enhanced by complement. We further tested the effect of the source of complement on the release of sVSG from *T. congolense* by using fresh mouse serum from either relatively resistant C57BL/6 mice or highly susceptible BALA/c mice. The results showed that, antibody-induced shedding of sVSG was higher in the presence of fresh C57BL/6 serum than in the presence of fresh BALB/c serum. Although this result could imply a beneficial effect of sVSG in resistance, other studies, as discussed in this thesis, indicate the opposite. All

these data suggest that the concentration of anti-VSG antibody, antibody class and complement can affect the release of sVSG from *T. congolense*.

4.2 Introduction

African trypanosomes are extracellular parasites causing sleeping sickness in humans and *nagana* in livestock. *Trypanosoma congolense* is the most important trypanosome species responsible for nagana in livestock (Hursey & Slingenbergh, 1995). The entire parasite surface is covered with a layer of a single species of glycoprotein, called variant surface glycoprotein (VSG) (Cross, 1990). Each parasite expresses about 10^7 densely packed identical VSG molecules on its surface (Cross, 1990, Gerold et al., 1996)). African trypanosomes have developed a very sophisticated mechanism of antigenic variation (Cross, 1990). The parasites will sequentially express a different VSG gene that is antigenically distinct from the previously expressed (El-Sayed et al., 2000, Ferrante & Allison, 1983). Antigenic variation permits the trypanosome population as a whole to keep "one step ahead"of the immune response. By this process, the parasites evade the immune system of their host to permit themselves to survive within the host (El-Sayed et al., 2000).

Molecules of VSG, the major component of the trypanosomal surface coat, are arranged in a tightly packed monolayer forming a 12-15 nm thick coat (Borst et al., 1998). They account for about 10% of the total amount of protein and are constitutively synthesized at a high rate (about 8% of total protein biosynthesis) (Kang et al., 2002). Each VSG monomer contains a N-terminal signal sequence and a hydrophobic C-terminal domain and is covalently attached to a glycosylphosphatidylinositol (GPI)

membrane anchor (Ferguson et al., 1985, Ferguson & Williams, 1988, Gerold et al., 1996, Kang et al., 2002). VSG coats are stably associated with the parasite plasma membrane but can be isolated as the membrane form of VSG (mfVSG) or the freely water-soluble glycoproteins (sVSG) after cell lysis. mfVSG is readily converted to sVSG during cell lysis by the action of an endogenous enzyme, the GPI-specific phospholipase C (GPI-PLC) (Ferguson, 1999). This GPI-PLC can cleave the GPI anchor, leaving the dimyristoylglycerol (DMG) compound of the mfVSG in the membrane, and release the glycosyl-inositol-phosphate (GIP)-VSG part (also called soluble VSG [sVSG]) (Fox et al., 1986, Magez et al., 1998).

There is growing evidence that VSG released from the parasite surface, is involved in affecting functions of macrophages, including the induction of synthesis of cytokines, such as TNF-α (Magez et al., 2002, Paulnock & Coller, 2001), and inhibiting IFN-γ-induced nitric oxide production (Coller et al., 2003). sVSG also has been found to induce polyclonal B cell activation (Diffley, 1983). The released sVSG from *T. brucei* can cause consumption of complement proteins, which may occur via the tremendous amounts of immune complexes generated during antibody-mediated clearance at each wave of parasitaemia (Musoke & Barbet, 1977). There is evidence that *T. congolense* bloodstream forms might evade complement lysis by shedding of immune complexes (Frevert & Reinwald, 1990). Several studies have also documented the immunostimulatory and regulatory activity of GPI anchors derived from *T. brucei* and other protozoa (Magez et al., 2002, Ropert & Gazzinelli, 2000, Tachado et al., 1997, Tachado & Schofield, 1994).

The release of VSG can be induced by hypotonic lysis (Cardoso De Almeida et al., 1999) or by different kinds of stress in *vitro* (Bowles & Voorheis, 1982, Rolin et al., 1996). In different strains of trypanosome-infected mice, the parasites are regularly confronted with the antibody specific for VSG and the complement cascades consisting of potentially different alleles of components. Antibody-induced release of sVSG has been reported (Liu et al., 1993). Within a narrow range of antibody concentration, the release of soluble VSG was correlated with the amount of antibody added to the mixture (Liu et al., 1993). There are no reports on the effect of antibody class or isotype regarding the release of sVSG from the trypanosomes, or the potential effects of different alleles of complement components.

In this study, we tried to get answers to the following questions: 1) Is the release of sVSG from *T. congolense* affected by the dosage or the class/isotype of antibody against VSG? 2) Does some released sVSG remain in a soluble complex with anti-VSG antibody? 3) Is the amount of sVSG released by antibody interaction affected by (a) complement and (b) by the source of complement? 4) Is some of the released sVSG present in a covalent complex with complement component C3b?

We incubated *T. congolense* with different amounts of IgM anti-VSG or IgG2a anti-VSG in the presence of 50% fresh mouse serum at 37^0C, and then tested the sVSG in the supernatants by western blot. We found that the release of sVSG occurs in an antibody dose-dependent pattern, and that more sVSG is released from *T. congolense* by interaction with IgM anti-VSG than by interaction with equal amounts of IgG2a anti-VSG. By immunprecipitation experiments, we also found that IgM anti-VSG forms soluble immune complexes with sVSG during the release from the surface of *T. congolense.* The results also show that antibody-induced release of sVSG occurs in the

absence of complement, but the release of sVSG can be enhanced by adding complement. We further tested the effect of the source of complement on the release of sVSG from *T. congolense. T. congolense* was incubated with anti-VSG antibody and fresh C57BL/6 serum or fresh BALB/c serum at 37^0C. The subsequent sVSG release into the culture supernatants of the incubation mixtures were tested by western blot. The results showed that, with the same amount of anti-VSG antibody, there was more sVSG released in the presence of fresh serum from C57BL/6 mice than in the presence of BALB/c serum. The biological significance of more sVSG being released by interaction with IgM anti-VSG, and in the presence of fresh C57BL/6 mouse serum will be discussed. These data suggest that the release of sVSG from the surface of *T. congolense* is affected in an antibody-dosage dependent pattern. The amount of sVSG released also depends on the antibody class and the source of complement.

4.3 Material and methods

Parasite

T. congolense, Trans Mara strain, variant antigenic type (VAT) TC13 was used in this study. The origin of this parasite strain has been previously described (Tabel, 1982). Frozen stabilates of parasites were used for infecting CD1 mice immunosuppressed with cyclophosphamide, and passages were made every third day as described previously (Tabel, 1982). The parasites purified from the blood of infected CD1 mice by DEAE-cellulose chromatography (Lanham & Godfrey, 1970).

Mice

Eight to 10 week-old, female, C57BL/6 and BALB/c mice and 5 to 8 week-old, female, Swiss white mice (CD1) were purchased from the Animal Resource Center of the University of Saskatchewan (Saskatoon, Canada). The mice were kept in polycarbonate cages on sawdust, and allowed free access to food and water throughout the experiments, according to the recommendations of the Canadian Council of Animal Care.

Fresh mouse serum and heat-inactivated mouse serum

Blood was collected from female 8 to 10 week-old C57BL/6 and BALB/c mice, and 5 to 8 week-old CD1 mice. The blood was allowed to clot and spun at 600×g at 4^0C to separate fresh serum. Serum samples were dispensed in aliquots and stored at -80^0C until use. Heat-inactivated mouse serum was obtained by incubating fresh CD1 mouse serum at 56^0C for 30 min.

Antibody

The production of the polyclonal rabbit anti-*T. congolense* (Shi et al., 2003), and of monoclonal antibodies (mAb) 6C1 (IgM), 5A8 (IgM) and 1D11 (IgG) specific for the VSG of VAT TC13 has been described (Wei et al., 1990). Peroxidase-labelled mouse anti-Rabbit IgG (H+L) was obtained from Jackson ImmunoResearch Lab. (West Grove, PA, USA). Mouse IgM (isotype control) was obtained from SeroTec Inc. (Raleigh, NC, USA). Goat anti-mouse IgM (μ chain-specific) was obtained from Cappel (MP Biomedicals, Irvine, CA, USA).

Buffer and Reagents

Tris-saline buffer with glucose (TSG) contained 17.5 g/L Tris (Sigma, Oakville, Ontario, Canada), 4.75 g/L Sodium Chloride (Sigma, Oakville, Ontario, Canada), 100 ml/L 1N hydrochloric acid and 12.5 g/L glucose (Sigma, Oakville, Ontario, Canada), pH 7.8. Phosphate buffered saline (PBS) contained 0.262 g/L sodium phosphate monobasic, 1.15 g/L sodium phosphate dibasic heptahydrate and 8.5 g/L sodium chloride, pH 7.2. Phosphate buffered saline with tween-20 (PBST) contained 0.5 ml/L Tween-20 (Sigma, Oakville, Ontario, Canada). BenchMark pre-stained protein ladder was obtained from Invitrogen Inc. (Burlington, Ontario).

Procedure of antibody-mediated release of sVSG

T. congolense were mixed with anti-VSG antibody (IgM or IgG) and 50% fresh mouse serum in TSG. *T. congolense* mixed with mouse IgM isotype control (25 μg/ml) and 50% fresh mouse serum in TSG, served as one control. *T. congolense* mixed with 50% fresh mouse serum in TSG without antibody, served as another control. The mixtures were incubated at 37^0C for 30 min, and then centrifuged at 13,000 x g at 4^0C for 15 min. The supernatants were collected and stored at -80^0C until analysis (modified from (Liu et al., 1993)). The kinetics of the release of sVSG with time of incubation was determined (Appendix 5).

Western blot for testing for sVSG

The sVSG-containing supernatants (described above) were separated by SDS-PAGE with 10% separating gels in a Mini-protean II apparatus (Bio-Rad, Richmond,

CA 94804). Samples diluted in non-reducing buffer were boiled for 5 min before loading onto the gel. Electrophoresis was carried out at 200 V until the dye marker reached the gel bottom. After SDS-PAGE was carried out, the protein bands were transferred from the gel to a nitrocellulose membrane in a Wet Transfer Cell (BIO-RAD Apparatus) at 75 V at 4^0C for 2 hr. The nitrocellulose membrane was then washed with PBS and blocked in 5% skim milk (DIFCO, Marland, USA) in PBS at 4^0C overnight. Then the nitrocellulose membrane was washed with PBST and incubated with rabbit anti-*T. congolense* antiserum in 2.5% skim milk (DIFCO) in PBST at RT for 1 hr. After washing, the last incubation was done with peroxidase-labelled mouse anti-rabbit IgG (H+L) at RT for 45 min. After washing again, the color development was carried out with HRP color development reagent. Partly purified sVSG was run with the samples as a positive control, which has been described (Liu et al., 1993). The results were scanned and the band densities were measured by AlphaImager 2000 software (Alpha Innotech Corporation, San Leandro, CA, USA).

Testing for the IgM anti-VSG/sVSG immune complexes

sVSG supernatants were prepared by interaction with *T. congolense*, IgM anti-VSG and fresh CD1 mouse serum as described above. The supernatant from IgM isotype control and negative control were also prepared as described above. One more control was added for testing whether IgM anti-VSG would bind to the released sVSG. The supernatant from the mixture of *T. congolense* and 50% fresh CD1 mouse was prepared following the protocol as described above, and then mixed with IgM anti-VSG (25 μg/ml) for another incubation at 37^0C for 30 min. The supernatant, which contain

sVSG and IgM anti-VSG antibody, was collected after centrifugation at 13,000 x g at 4^0C for 15 min. The parasites were discarded with the pellet.

All the supernatants were mixed with goat anti-mouse IgM in the presence of 10 mM EDTA at RT for 1 hr. The mixtures were then centrifuged at 13,000 x g at 4^0C for 15 min. The precipitates were washed with PBS and then suspended in PBS/1% SDS. The precipitates were then analyzed by western blot with rabbit anti-*T. congolense* antiserum (as western blot for testing sVSG).

Statistical analysis

Data are presented as means ± standard error (SE). Analysis of Variance (ANOVA) was carried out using Excel software (Microsoft, Santa Monica, CA, USA). A P value < 0.05 was considered statistically significant.

4.4 Results

The anti-VSG induced release of sVSG from T. congolense depends on antibody concentration

First we tested whether the release of sVSG from *T. congolense* was affected by the anti-VSG antibody dosage. *T. congolense* were incubated at 37^0C for 30 min with different amounts of anti-VSG antibody (IgM 6C1 or IgG2a 1D11) in the presence of 50% fresh mouse serum from CD1, C57BL/6 or BALB/c mice. The supernatants were collected and tested by western blot with rabbit anti-*T. congolense* antiserum. We found that the parasites released more sVSG when the antibody concentration was increased. This happened when the parasites were allowed to interact with either IgM anti-VSG (6C1) or IgG2a anti-VSG (1D11) in the presence of different sources of fresh mouse

serum (Fig 4.1, 4.2, 4.3). These data indicate that the release of sVSG from *T. congolense* occurs in an antibody dose-dependent pattern.

The results also show that two bands of higher molecular weight could be detected in the samples prepared in the presence of anti-VSG antibody and fresh mouse serum but could not be detected in the absence of anti-VSG antibody. The band density of these two bands increased with the IgM anti-VSG concentration. The molecular weights of these two bands were about 210 kD and 105 kD respectively. These two bands could be other parasite antigen released by complement-mediated lysis. On the other hand, they could be covalently bound complexes of sVSG, and degradation products of complement component C3.

FIGURE 4.1

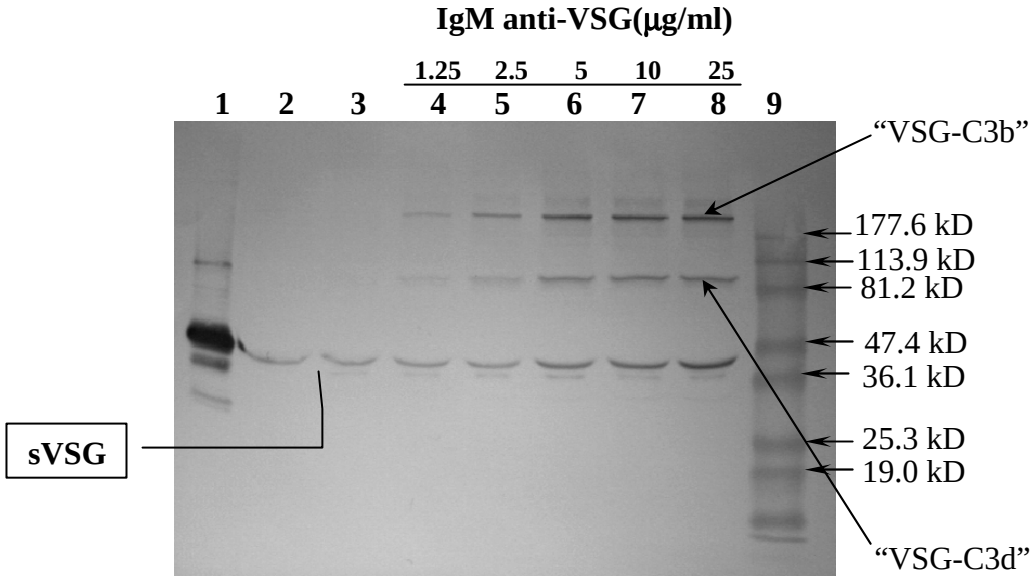


FIGURE 4.1 IgM anti-VSG induces the release of sVSG from *T. congolense.* In the presence of 50% fresh CD1 mouse serum, *T. congolense* (10^8/ml) were mixed with different amounts of IgM anti-VSG (mAb 6C1). The mixtures were incubated at 37^0C for 30 min, and then spun down 15 min at 13,000 g at 4^0C for separating the supernatant. The supernatants were tested for sVSG by Western blot ($1^{\#}$ Ab: rabbit anti-T. congolense antiserum, $2^{\#}$ Ab: peroxidase-labelled mouse anti-rabbit IgG (H+L)).

Lane 1: partly purified sVSG

Lane 2: mouse IgM (isotype control) + *T. congolense* +fresh mouse serum

Lane 3: No antibody + *T. congolense* + fresh mouse serum

Lane 4: IgM anti-VSG1.25 μg/ml + *T. congolense* + fresh mouse serum

Lane 5: IgM anti-VSG 2.5 μg/ml + *T. congolense* + fresh mouse serum

Lane 6: IgM anti-VSG 5 μg/ml + *T. congolense* + fresh mouse serum

Lane 7: IgM anti-VSG 10 μg/ml + *T. congolense* + fresh mouse serum

Lane 8: IgM anti-VSG 25 μg/ml + *T. congolense* + fresh mouse serum

Lane 9: BenchMark prestained protein ladder.

FIGURE 4.2

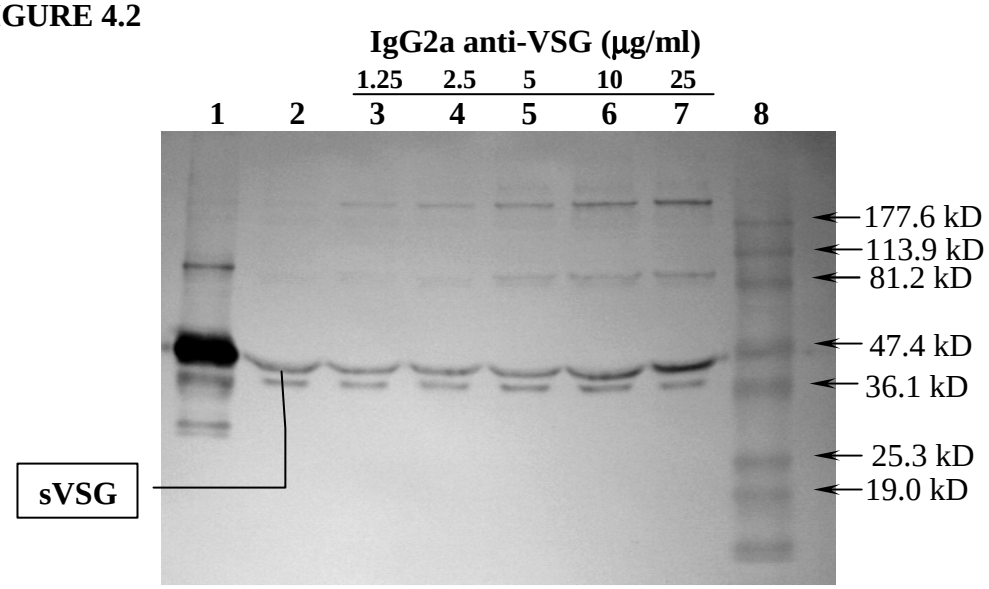


FIGURE 4.2 IgG anti-VSG induces the release of sVSG from *T. congolense.* In the presence of 50% fresh CD1 mouse serum, *T. congolense* (10^8/ml) were mixed with different amounts of IgG anti-VSG (mAb 1D11). The mixtures were incubated at 37^0C for 30 min, and then spun down 15 min at 13,000 g at 4^0C for separating the supernatant. The supernatant were tested for sVSG by Western blot ($1^{\#}$ Ab: rabbit anti-T. congolense antiserum, $2^{\#}$ Ab: peroxidase-labelled mouse anti-rabbit IgG (H+L)).

Lane 1: partly purified sVSG

Lane 2: No antibody + *T. congolense* + fresh mouse serum

Lane 3: IgG2a anti-VSG1.25 μg/ml + *T. congolense* +fresh mouse serum

Lane 4: IgG2a anti-VSG 2.5 μg/ml + *T. congolense* +fresh mouse serum

Lane 5: IgG2a anti-VSG 5 μg/ml + *T. congolense* +fresh mouse serum

Lane 6: IgG2a anti-VSG 10 μg/ml + *T. congolense* +fresh mouse serum

Lane 7: IgG2a anti-VSG 25 μg/ml + *T. congolense* +fresh mouse serum

Lane 8: BenchMark prestained protein ladder.

FIGURE 4.3

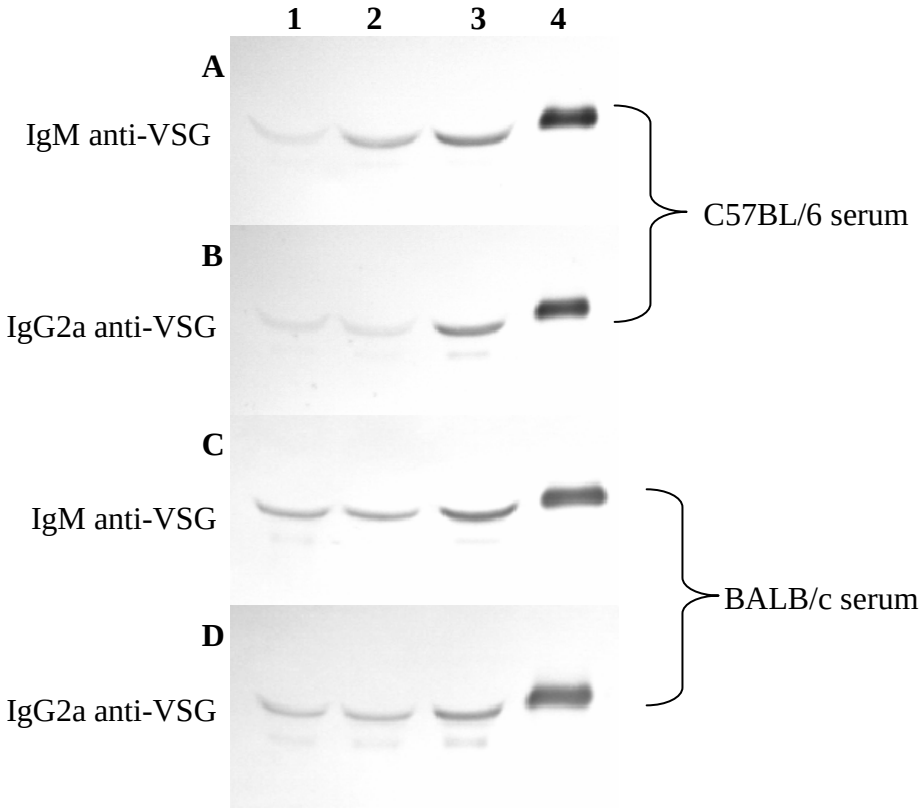


FIGURE 4.3 Antibody induces the release of sVSG from *T. congolense* in the presence of fresh C57BL/6 or BALB/c mouse serum. *T. congolense* (10^7/ml) were incubated with **(A)** IgM anti-VSG (mAb 6C1) and 50% fresh C57BL/6 serum; **(B)** IgG2a anti-VSG (mAb 1D11) and 50% fresh C57BL/6 serum; **(C)** IgM anti-VSG (mAb 6C1) and 50% fresh BALB/c serum; **(D)** IgG2a anti-VSG (mAb 1D11) and 50% fresh BALB/c serum were tested by Western blot ($1^{\#}$ Ab: rabbit anti-*T. congolense* antiserum, $2^{\#}$ Ab: peroxidase-labelled mouse anti-rabbit IgG (H+L)). The set of results shown is representative of three similar experiments.

Lane 1: No antibody + *T. congolense* + fresh mouse serum

Lane 2: anti-VSG 1.25 µg/ml + *T. congolense* + fresh mouse serum

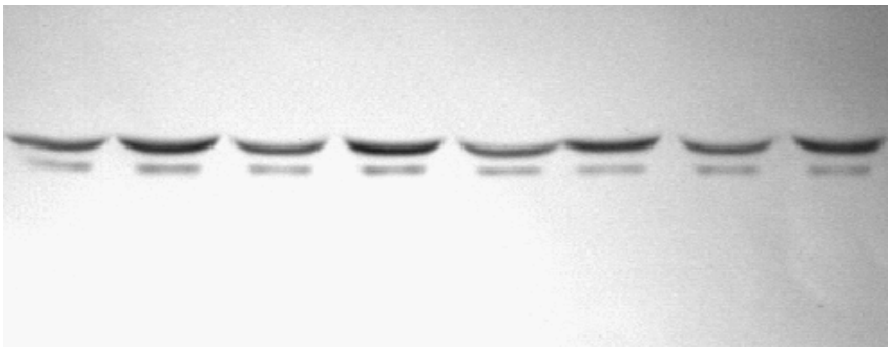
Lane 3: anti-VSG 5 µg/ml + *T. congolense* + fresh mouse serum

Lane 4: partly purified sVSG

IgM anti-VSG is more efficient than IgG2a anti-VSG in inducing shedding of sVSG from T. congolense

Then, we assessed whether the class of antibody would affect the efficiency of release of sVSG from *T. congolense*. The supernatants from *T. congolense* were incubated with equal amounts of IgM anti-VSG or IgG2a anti-VSG in the presence of fresh CD1 serum. The supernatants of the mixtures were tested for sVSG by western blot with rabbit anti-*T. congolense* antiserum. We measured the band densities by Alphaimage software. The results showed that, in the presence of fresh mouse serum, there was more sVSG released from *T. congolense* by interaction with IgM anti-VSG than by interaction with IgG2a anti-VSG (band densities: 448.9 ± 40.8 vs 295.1 ± 14.3, respectively. p=0.012) (Fig 4.4). These results indicate that IgM anti-VSG is more efficient than IgG2a anti-VSG in inducing *T. congolense* to release sVSG.

FIGURE 4.4



IgM anti-VSG	-	+	-	+	-	+	-	+
IgG2a anti-VSG	+	-	+	-	+	-	+	-
Band density	**295**	**540**	**313**	**486**	**316**	**414**	**254**	**354**

FIGURE 4.4 IgM anti-VSG induces the release of more sVSG from *T. congolense* than IgG2a anti-VSG. The sVSG supernatant form the culture of *T. congolense* (10^8/ml) mixed 50% of fresh CD1 mouse serum and same amount of IgM anti-VSG (mAb 6C1) (25 μg/ml) or IgG2a anti-VSG (mAb 1D11) (25 μg/ml) were tested by Western blot ($1^{\#}$ Ab: rabbit anti-*T. congolense* antiserum, $2^{\#}$ Ab: peroxidase-labelled mouse anti-rabbit IgG (H+L)). The band densities were measured by Alphaimager software, and shown in the bottom line. The mean band density of sVSG released by interacted with IgM anti-VSG is 448.9 ± 40.8, and by interacted with IgG2a anti-VSC is 295.1 ± 14.3 (p=0.012). The set of results shown is representative of two similar experiments.

After interaction with T. congolense, some IgM anti-VSG remains in a soluble complex with the shed sVSG

We further tested whether IgM anti-VSG can occur in a soluble immune complex with sVSG after sVSG had been released from *T. congolense*. *T. congolense* was incubated with fresh mouse serum and IgM anti-VSG antibody and the supernatants were subjected to immunoprecipitation by anti-mouse IgM antibody. The precipitates were tested for sVSG by western blot with rabbit anti-*T. congolense* antiserum. No sVSG was detectable in the precipitate of two different control preparations: (a) supernatant derived from mixture of *T. congolense* and fresh CD1 mouse serum (Fig. 4.5 lane 1) and (b) supernatant of (a) that was spiked with IgM anti-VSG (Fig. 4.5 lane2). A strong band of sVSG could be detected in the precipitate obtained from the supernatant of incubated mixtures of *T. congolense,* IgM anti-VSG and fresh mouse serum (Fig. 4.5, lane 3). A very faint band of sVSG was detectable in the supernatant derived from the incubated mixture of *T. congolense*, 25 μg/ml mouse IgM (normal antibody control) and fresh CD1 mouse serum (Fig 4.5 lane 4). These data indicate that IgM anti-VSG, after interaction with the surface of *T. congolense,* can remain in a soluble complex with sVSG when sVSG was released. The result obtained with the normal antibody control suggests that some natural IgM might weakly react with VSG of *T. congolense* and induce the release of very low amount of sVSG and remain bound to sVSG.

FIGURE 4.5

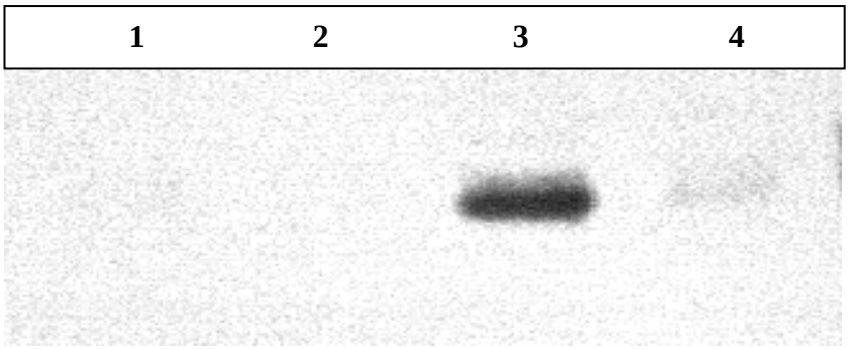


FIGURE 4.5 Some IgM anti-VSG remains in a soluble immune complex with sVSG after sVSG is released from the surface of *T. congolense.* IgM/anti-IgM immunoprecipitations were carried out using the supernatants from the following incubated mixtures: The set of results shown is representative of two similar experiments.

Lane 1: *T. congolense* (10^8/ml) + 50% fresh CD1 mouse serum (control)

Lane 2: Like above, but after incubation and centrifugation, 25 μg/ml IgM anti-VSG was added to the supernatant (control)

Lane 3: *T. congolense* (10^8/ml) + 50% fresh CD1 mouse serum + 25 μg/ml IgM anti-VSG

Lane 4: *T. congolense* (10^8/ml) + 50% fresh CD1 mouse serum + 25 μg/ml mouse IgM

The precipitates from the immunoprecipitation were tested for sVSG by western blot ($1^{\#}$ Ab: rabbit anti-*T. congolense* antiserum, $2^{\#}$ Ab: peroxidase-labelled mouse anti-rabbit IgG (H+L)).

Antibody against VSG can induce release of sVSG in the absense of complement activity

We tested the ability of anti-VSG antibody to induce the release of sVSG from *T. congolense* in the absence of active complement by using heat-inactivated (56^{o}C / 30 min) CD1 mouse serum. *T. congolense* (10^{8}/ml) was incubated with different amounts of IgM anti-VSG (mAb 6C1) in the presence of 50% heat-inactivated CD1 mouse serum. The supernatant of the incubated mixtures were tested for sVSG by western blot with rabbit anti-*T. congolense* antiserum. We found that, without active complement, the parasites released more sVSG with increasing antibody concentrations (figure 4.6)**.** Antibody-induced release of sVSG from *T. congolense* can occur independently of complement. Antibody-induced release of sVSG in the absence of complement also proceeds in a dose- dependent pattern (figure 4.6).

FIGURE 4.6

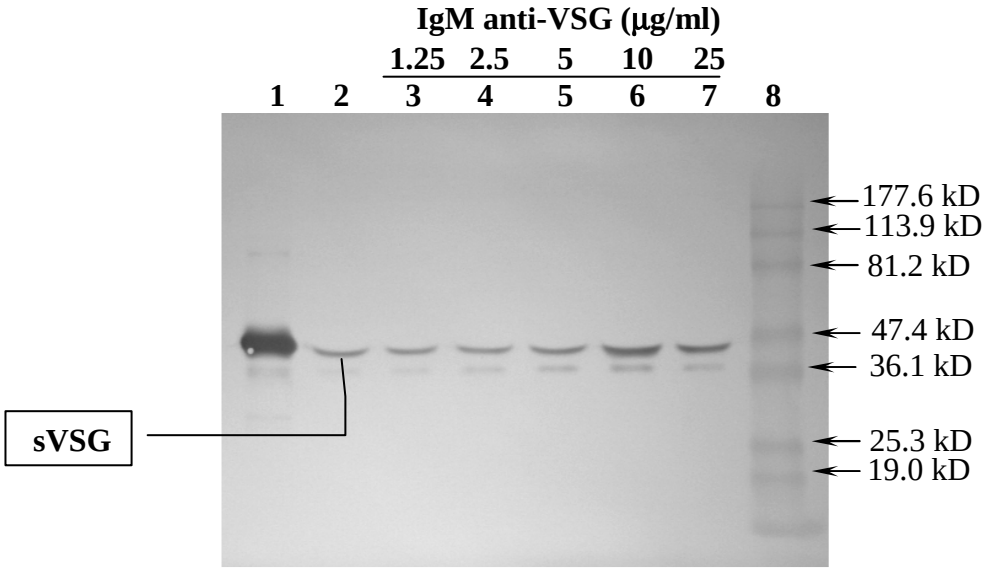


FIGURE 4.6 IgM anti-VSG induces the release of sVSG from *T. congolense* in the absence of fresh mouse serum. In the presence of 50% heat-inactivated CD1 mouse serum, *T. congolense* (10^8/ml) were mixed with different amount of IgM anti-VSG (mAb 6C1). The mixture was incubated at 37^0C for 30 min, and then spun down 15 min at 13,000 x g at 4^0C for separating the supernatant. The sVSG in the supernatant were tested by Western blot ($1^{\#}$ Ab: rabbit anti-*T. congolense* antiserum, $2^{\#}$ Ab: peroxidase-labelled mouse anti-rabbit IgG (H+L)).

Lane 1: partly purified sVSG

Lane 2: No antibody + *T. congolense* + heat-inactivated mouse serum

Lane 3: IgM anti-VSG1.25 μg/ml + *T. congolense* + heat-inactivated mouse serum

Lane 4: IgM anti-VSG 2.5 μg/ml + *T. congolense* + heat-inactivated mouse serum

Lane 5: IgM anti-VSG 5 μg/ml + *T. congolense* + heat-inactivated mouse serum

Lane 6: IgM anti-VSG 10 μg/ml + *T. congolense* + heat-inactivated mouse serum

Lane 7: IgM anti-VSG 25 μg/ml + *T. congolense* + heat-inactivated mouse serum

Lane 8: BenchMark prestained protein ladder.

Antibody-induced shedding of sVSG from T. congolense is higher in the presence of serum complement

To assess whether the source of complement would affect the release of sVSG from *T. congolense*, complement from C57BL/6, BALB/c and CD1 mice were used. *T. congolense* (10^8/ml) was incubated with IgM anti-VSG (mAb 6C1: 25 μg/ml) in the presence of 50% fresh mouse serum with or without 10 mM EDTA (an inhibitor of complement activation). The supernatants from the incubated mixtures were tested for sVSG by western blot with rabbit anti-*T. congolense* antiserum. The results showed that more sVSG was released from *T. congolense* by IgM anti-VSG in the presence of fresh mouse serum than in the presence of EDTA/fresh mouse serum (Fig. 4.7). These data indicate that antibody-induced release of sVSG can occur without complement (presence of EDTA), but the complement activation enhances the release of sVSG from the parasites. Two bands of higher molecular weight, which have beed discussed above (Fig 4.1), could be detected in samples prepared in the presence of fresh mouse serum without EDTA but could not be detected in samples prepared in the presence of fresh mouse serum with EDTA.

FIGURE 4.7

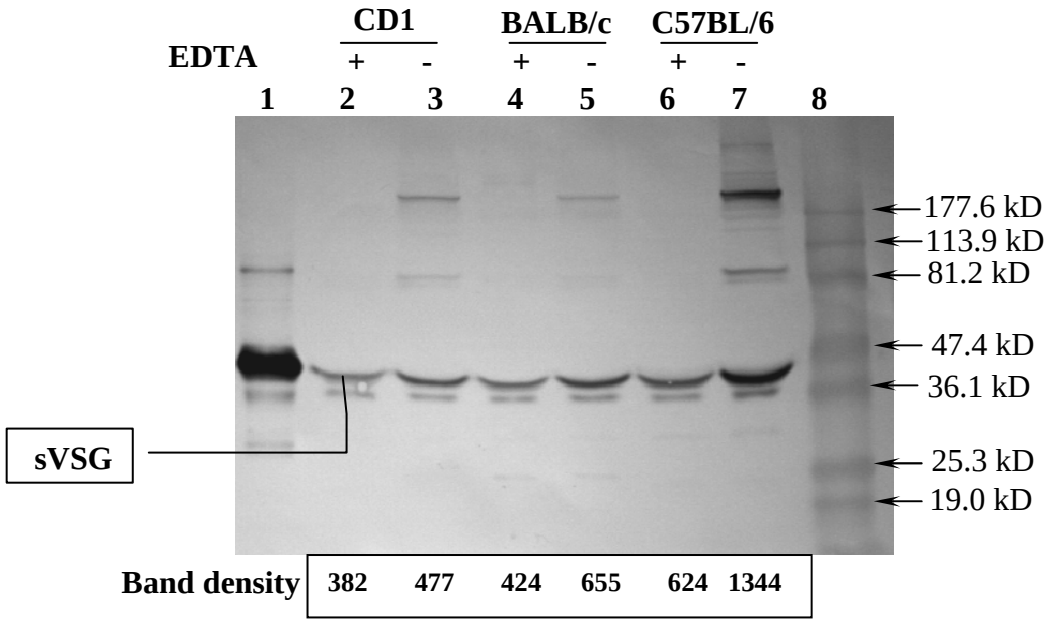


FIGURE 4.7 More IgM anti-VSG-induced release of sVSG from *T. congolense* was found in the presence than in the absence of complement activation, and fresh serum from C57BL/6 mice is more effective in enhancing antibody-induced release of sVSG than fresh serum from BALB/c or CD1 mice. *T. congolense* (10^8/ml) was incubated with IgM anti-VSG (mAb 6C1 10 μg/ml) and 50% fresh mouse serum with or without 10 mM EDTA. The supernatant of the mixtures were tested for sVSG by Western blot ($1^{\#}$ Ab: rabbit anti-*T. congolense* antiserum, $2^{\#}$ Ab: peroxidase-labelled mouse anti-rabbit IgG (H+L)). The density of sVSG bands were measured by Alphaimager software, and are showed in the bottom line.

Lane 1: partly purified sVSG

Lane 2: fresh CD1 serum/10 mM EDTA + *T. congolense* (10^8/ml) + IgM anti-VSG

Lane 3: fresh CD1 serum + *T. congolense* (10^8/ml) + IgM anti-VSG

Lane 4: fresh BALB/c serum/10 mM EDTA + *T. congolense* (10^8/ml) + IgM anti-VSG

Lane 5: fresh BALB/c serum + *T. congolense* (10^8/ml) + IgM anti-VSG

Lane 6: fresh C57BL/6 serum/10 mM EDTA + *T. congolense* (10^8/ml) + IgM anti-VSG

Lane 7: fresh C57BL/6 serum + *T. congolense* (10^8/ml) + IgM anti-VSG

Lane 8: BenchMark prestained protein ladder.

Fresh serum of C57BL/6 mice enhances antibody-induced shedding of sVSG more than fresh serum from BALB/c mice

T. congolense were incubated with different amounts of IgM anti-VSG in the presence of 50% fresh mouse serum from either C57BL/6 or BALB/c mice. The supernatants from the incubated mixtures were tested for sVSG by western blot. The results showed that *T. congolense* released more sVSG by interaction with IgM anti-VSG in the presence of fresh C567BL/6 serum than in the presence of fresh BALB/c serum or CD1 serum (Fig. 4.7, 4.8). Similar results were found by using IgG anti-VSG (Appendix 9.7). These findings indicate that the source of complement can affect the antibody-induced shedding of sVSG from *T. congolense*. Complement from the relatively resistant C57BL/6 mice is more effective than complement from the susceptible BALB/c mice in enhancing the antibody-induced release of sVSG.

FIGURE 4.8

IgM anti-VSG	5	5	2.5	2.5	0	0
C57BL/6 serum	+	-	+	-	+	-
BALB/c serum	-	+	-	+	-	+
Band density	**515**	**315**	**187**	**78**	**28**	**68**

FIGURE 4.8 More sVSG is released from *T. congolense* by interaction with IgM anti-VSG in the presence of fresh C57BL/6 serum than in the presence of fresh BALB/c serum. *T. congolense* (10^7/ml) was incubated with different amounts of IgM anti-VSG (mAb 6C1) and 50% fresh C57BL/6 serum or fresh BALB/c serum. The supernatant of the mixtures were tested for sVSG by Western blot ($1^{\#}$ Ab: rabbit anti-*T. congolense* antiserum, $2^{\#}$ Ab: peroxidase-labelled mouse anti-rabbit IgG (H+L)). The band densities were measured by Alphaimage software. The set of results shown is representative of two similar experiments.

4.5 Discussion

Since African trypanosomes are extracellular parasites, any sVSG shed from the parasites are released into the circulation. sVSG has been found in the plasma of mice infected with *T. brucei* (Diffley et al., 1980, Paulnock & Coller, 2001, Weitz, 1960) and *T. congolense* (Tabel unpublished). The immune system of an infected mammalian host is regularly confronted with these circulating sVSG (Magez et al., 2002). GPI-PLC-mediated VSG release is part of the parasite biology (Black et al., 1982, Seyfang et al., 1990, Shapiro, 1986). In the infected mice, the trypanosomes are faced with the antibody specific to VSG and the complement system. We tested the effect of the class of antibody against VSG, the influence of complement, and the potential source of complement on the release of sVSG from *T. congolense* by *in vitro* experiments. The use of 50% of fresh mouse serum mimicked the *in vivo* condition as closely as possible.

In this study, the results showed that, the antibody-induced release of sVSG from *T. congolense* occurs in a dose-dependent pattern (Fig. 4.1, 4.2, 4.3), and includes both a complement-independent and -dependent pathway (Fig. 4.6, 4.7). Our results showed that the antibody-induced complement-independent release of sVSG occurs in a dose-dependent pattern (Fig. 4.6), and complement activation enhances the antibody-induced release of sVSG from *T. congolense* (Fig. 4.7).

The results also showed that IgM anti-VSG is more effective in inducing release sVSG than IgG2a anti-VSG (Fig. 4.4). This may directly be due to the 10 valent binding potential of one molecule of IgM anti-VSG onto the VSG epitopes exposed at the surface of the parasite. Alternatively, it may be indirectly due to IgM being more efficient in inducing complement-mediated parasite lysis. It has been reported that cross

linking of VSG molecules, such as trypanosomes treated with IgG anti-VSG, caused the rapid accumulation of ligands along the line of flagellar attachment and their shedding by formation of coat-covered vesicles and filopodia (Frevert & Reinwald, 1988, Shakibaei & Frevert, 1992). IgM antibodies, after binding to antigen, are most efficient in activating the complement cascade (Law & Reid, 1988). Our data also showed that some IgM anti-VSG remains in a soluble immune complex with sVSG after the sVSG has been released from the surface of *T. congolense* (Fig 4.5). It has previously been suggested that shedding of immune complexes might be a method by which trypanosomes evade complement-mediated lysis (Frevert & Reinwald, 1990). IgM antibodies are the first and predominant class of anti-trypanosomal antibodies in African trypanosomes infections (Binz et al., 1968, Clarkson, 1976, Greenwood & Whittle, 1973, Hudson et al., 1976, Luckins, 1976). IgM anti-VSG is equally efficient in inducing phagocytosis of *T. congolense* by macrophages as IgG2a anti-VSG (Kaushik et al., 1999, Shi et al., 2004). Here, we show that IgM anti-VSG is more efficient in inducing release of sVSG from the parasites. sVSG has been shown to have detrimental effects in trypanosomal infections. sVSG has been shown to induce polyclonal B cell activation (Diffley, 1983). sVSG can activate macrophages to produce TNF-α (Magez et al., 2002, Paulnock & Coller, 2001), which plays an important role in the immunopathology of African trypanosomiasis (Hunter et al., 1991, Magez et al., 1999, Magez et al., 2002, Okomo-Assoumou et al., 1995, Sileghem et al., 1994b). sVSG also has the ability to inhibit IFN-γ-induced production of nitric oxide (Coller et al., 2003) which is trypanotoxic (Duleu et al., 2004, Gobert et al., 1998, Kaushik et al., 1999, Sternberg et al., 1994, Vincendeau & Daulouede, 1991). All these observations suggest that IgM anti-

VSG antibody, although being able to mediate phagocytosis and lysis of the parasite, also mediates detrimental effects by its ability to induce the release of sVSG from the parasites.

In *T. congolense* infection, BALB/c mice are highly susceptible whereas C57BL/6 mice are relatively resistant (Ogunremi & Tabel, 1995, Pinder, 1984). After infection with 10^3 *T. congolense*, the mean survival time of BALB/c mice is 8.4±0.5 days, but the C57BL/6 mice control the first peak of parasitemia and survive for more than 5 months (Ogunremi & Tabel, 1995). Our data showed that, after binding of anti-VSG antibody to *T. congolense*, there was more sVSG released in the presence of fresh C57BL/6 serum than in the presence of fresh BALB/c serum (Fig 4.7). It was also found in our lab that, in the presence of anti-VSG antibody and *T. congolense*, more complement-mediated lysis of *T. congolense* occurred with fresh C57BL/6 serum than with fresh BALB/c serum (data not shown). Therefore, more sVSG released in the presence of fresh C57Bl/6 serum might be due to the fact that more complement-mediated parasite lysis occurs with fresh C57BL/6 serum than with fresh BALB/c serum. This conclusion is in line with the previous observation that the complement cascade of the relatively resistant C57Bl/6 mice allowed deposition of significantly more C3 fragments onto *T. congolense* than the complement cascade of the susceptible BALB/c (Fig. 3.1). This observation suggests that genetic polymorphism may affect the effiency of complement activation, such that the complement cascade of C57BL/6 and BALB/c mice is different and differently affects parasite clearance. The more efficient activation of the complement cascade in *T. congolense* infected C57BL/6 mice might, however, have diverse effects on controlling the disease. The enhanced release of sVSG in the presence of fresh C57BL/6 serum might have a negative effect on controlling infections.

It has been observed that sVSG induces TNF-α synthesis in macrophages (Paulnock & Coller, 2001) and inhibits synthesis of NO by IFN-γ-stimulated macrophages (Coller et al., 2003). On the other hand, this negative effect might be outweighed by C57BL/6 mice being more efficient in inducing more complement-mediated lysis of the parasite.

In conclusion, the results shown here indicate that antibody-induced release of sVSG occurs in a dose-dependent pattern. There is more sVSG released from *T. congolense* by interaction with IgM anti-VSG than by interaction with equal amounts of IgG anti-VSG. The antibody-induced release of sVSG from *T. congolense* occurs in the absence of complement, but is enhanced by the presence of complement. The data also show that complement from relatively resistant C57BL/6 mice is more effective than complement from the susceptible BALB/c mice in enhancing the antibody-induced release of sVSG. All these data suggest that the release of sVSG from *T. congolense* is affected by the dosage and the class of the anti-VSG antibody, and also affected by the source of complement.

5. GENERAL DISSCUSSION AND CONCLUSION

The aim of this thesis work was to test the effect of IgM anti-VSG on *T. congolense*. It was shown that, in the presence of fresh mouse serum, the interaction of IgM anti-VSG with *T. congolense* could cause several immune reactions.

It had previously been observed that IgM anti-VSG can mediate phagocytosis of parasites by macrophages in vitro and in vivo (Kaushik et al., 1999, Shi et al., 2004). The receptor(s) on the macrophages involved in IgM anti-VSG-mediated phagocytosis of African trypanosomes has been unknown.

The present study shows that CR3 (CD11b/CD18) is the major receptor involved in IgM anti-VSG mediated phagocytosis of *T. congolense*. The IgM anti-VSG-mediated phagocytosis of *T. congolense* via CR3 is entirely complement-dependent. The signaling via CR3, associated with IgM anti-VSG-mediated phagocytosis of *T. congolense*, either directly or indirectly enhances the TNF-α production, but inhibits NO synthesis.

The biological significance of CR3 in *T. congolense* infections appears to be multi-faceted. The function of CR3 in *T. congolense* infections is beneficial in controlling the parasitemia through its ability to mediate phagocytosis of IgM anti-VSG-coated parasites. On the other hand, the modulation of macrophage functions subsequent to phagocytosis of trypanosomes has to be taken into account. There is evidence that high levels of TNF-α produced by macrophages contribute to disease in African trypanosomiasis (Hunter et al., 1991, Magez et al., 1999, Magez et al., 2002, Okomo-Assoumou et al., 1995, Sileghem et al., 1994b). NO secreted by macrophages appears to

be beneficial to the host since it is cytotoxic for extracellular trypanosomes (Duleu et al., 2004, Gobert et al., 1998, Kaushik et al., 1999, Vincendeau & Daulouede, 1991, Vincendeau et al., 1992). It has been shown in this thesis that phagocytosis of trypanosomes via CR3 inhibits NO synthesis. It is presently not clear what the net effects of the functions of CR3 are in *T. congolense* infections. I suggest that the overall contribution of CR3 signaling might, especially in the susceptible host, be negative rather than positive. This tentative conclusion requires confirmation by in vivo experiments. Other receptors involved in this process are presently unknown. The mannose receptor (Linehan et al., 2000) and Toll-like receptors (Campos et al., 2001) are possible candidates.

The release of sVSG from *T. congolense* by the interaction of IgM anti-VSG with parasites was studied. The data of this study show that IgM anti-VSG induces the release of sVSG from *T. congolense* more effectively than IgG anti-VSG. Some IgM anti-VSG persists in a soluble immune complex with sVSG. The antibody-induced release of sVSG can occur without complement, and is enhanced by complement. Anti-VSG antibody-mediated release of sVSG from *T. congolense* is more enhanced in the presence of complement from C57BL/6 mice than the complement from BALB/c mice.

The parasitemia in African trypanosomiasis is believed be controlled by three different mechanisms: (1) antibody/complement-mediated lysis (Flemmings & Diggs, 1978), (2) antibody-mediated phagocytosis (Tabel et al., 2000) and (3) trypanotoxic NO secreted by macrophages after antibody-mediated phagocytosis (Duleu et al., 2004, Gobert et al., 1998, Kaushik et al., 1999, Vincendeau & Daulouede, 1991).

The biological role of IgM anti-VSG in the disease is complex. IgM anti-VSG contributes to the control of the *T. congolense* infection by mediating phagocytosis and

lysis of the parasite. The beneficial effects of lysis and phagocytosis of the parasite might be outweighed by the ability of IgM anti-VSG to mediate enhanced synthesis of TNF-α but decreased synthesis of trypanotoxic NO. In addition, excessively shed sVSG might bind newly synthesized anti-VSG and thus prevent this newly produced antibody from mediating lysis and phagocytosis of the parasite.

The data of this study also show that complement plays an important role in IgM anti-VSG-mediated phagocytosis of *T. congolense* as well as in the antibody-induced release of sVSG from *T. congolense*. Complement contributes to control the parasitemia by antibody/complement-mediated lysis and by complement component iC3b-mediated phagocytosis of IgM antibody-coated trypanosomes. On the other hand, complement enhances the release of sVSG from trypanosomes. Soluble VSG has been shown to be contributing to the detrimental effects in trypanosome infection by enhancing synthesis of TNF-α (Paulnock & Coller, 2001) but inhibiting synthesis of NO (Campos et al., 2001, Coller et al., 2003, Magez et al., 2002).

Several differences have been found between the complement from relatively resistant C57BL/6 and highly susceptible BALB/c mice. Firstly, under conditions that allowed activation of the alternative pathway of complement, more C3 fragments were deposited onto zymosan in the presence of fresh serum from C57Bl/6 than that of BALB/c mice (Ogunremi et al., 1993). Secondly, when incubated with IgM anti-VSG, more complement C3 fragments were deposited onto *T. congolense* in the presence of fresh C57BL/6 serum than in the presence of fresh BALB/c serum (Fig.3.1). Thirdly, complement from C57BL/6 mice is more efficient in enhancing the anti-VSG antibody-mediated release of sVSG from *T. congolense* than complement from BALB/c mice

(Fig.4.7, 4.8). Fourthly, more IgM anti-VSG/complement-mediated lysis of *T. congolense* occurs in the presence of fresh C57BL/6 serum than in the presence of fresh BALB/c serum (Tabel, unpublished.).

Deposition of more C3 fragments onto *T. congolense* will contribute to controlling the parasitemia by IgM antibody-mediated phagocytosis via CR3. However, as discussed above, the modulation of macrophages associated with CR3-mediated phagocytosis inhibits synthesis of NO by these cells and presumably reduces the potential control of parasitemia by NO. It was also shown that deposition of C3 fragments onto zymosan has a low, but statistically significant, negative correlation with the survival of F2 mice [BALB/c X C57BL/6] infected with *T. congolense*. Therefore, the overall contribution of complement to resistance and pathogenesis of trypanosomiasis is complex. Whether the superior trypanolytic activity of complement of the relatively resistant C57BL/6 mice might represent an innate factor of resistance against infection and/or disease remains to be determined. If so, there should be one or more relevant differences in complement genes of C57BL/6 and BALB/c mice. Such gene(s) should be located within one of the identified quantitative trait loci (Kemp et al., 1997).

6. FUTURE DIRECTIONS

Based on the studies in this thesis, many questions have been brought up that, if answered, would shed more light on the role of IgM anti-VSG in *T. congolense* infections.

(1) The role of NO in controlling the parasitemia in vivo. The signaling via CR3, associated with IgM anti-VSG-mediated phagocytosis, inhibits NO synthesis. NO has been shown to be trypanostatic for *T. congolense*, *T. musculi*, *T. gambiense* and *T. brucei* in vitro (Kaushik et al., 1999, Vincendeau & Daulouede, 1991, Vincendeau et al., 1992). These results beg the question: does NO contribute to controlling the parasitemia in vivo? The question could be answered by infecting nitric oxide synthase-deficient mice. Also, arginase activity, which is competing with the iNOS pathway, should be measured (Duleu et al., 2004).

(2) The receptors for IgM anti-VSG-mediated phagocytosis of *T. congolense* other than CR3. CR3 deficiency is not associated with a complete lack of IgM anti-VSG-mediated phagocytosis of *T. congolense*, which means other receptors must be involved in this process. The relevant receptors are presently unknown. Are the mannose receptor (Linehan et al., 2000) and Toll-like receptor (Campos et al., 2001) possible candidates? The potential role of mannose receptor in phagocytosis of *T. congolense* could be detected by using soluble mannan, which can block the function of mannose receptor (Karaca et al., 1995).

(3) In the experiments concerned with the release of sVSG, two top bands could always be detected by western blot (Fig.4.1, 4.2, 4.7). They could be detected in samples prepared in the presence of fresh mouse serum and anti-VSG antibody, but could not be detected in samples prepared in the presence of heat-inactivated serum or EDTA/fresh mouse serum. The molecular weights of these two bands were about 210 kD and 105 kD respectively. These two bands could be other parasite antigen released by complement-mediated lysis. On the other hand, they could be complexes of sVSG covalently bound to degradation products of complement component C3, such as VSG-C3b and VSG-C3d. These complexes might have biological significance. It has been reported that antigen covalently bound to C3d is up to 1000-fold more immunogenic than native antigen (Fearon & Carroll, 2000). Are these two bands in fact complexes of VSG and C3 fragments? This could be tested by anti-C3 antibody.

(4) This investigation did not in any way clarify the mechanism of IgM-antibody-mediated release of sVSG. It has been reported that anti-trypanosomal antibodies induce the formation and shedding of VSG-coated filopodia (Shakibaei & Frevert, 1992). Dose IgM anti-VSG antibody induce the formation and release of VSG-coated filopodia?

7. REFERENCES

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8. APPENDIX

8.1 IgM anti-VSG-mediated phagocytosis of *Trypanosoma congolense* by a macrophage cell line occurs in an antibody dose-dependent pattern

Aim

To test whether the degree of phagocytosis of *T. congolense* by macrophages correlates with the concentration of IgM anti-VSG antibody

Protocol

1. Cells of macrophage cell line BALB.BM were seeded in Lab-Tek 16-chamber glass slides (Electron Microscopy Sciences, Washington, USA) at 5×10^4 cells per well in 200 µl DMEM complete medium with 12 ng/ml IFN-γ (R&D systems, Minneapolis, USA) for 24 hr at 37^0C.
2. The cells were washed 2x with DMEM. The mixture of freshly isolated *T. congolense* (macrophage: trypanosome ratio = 1:10) with 50% fresh CD1 mouse serum and different amounts of IgM anti-VSG were added to the cells and cultured for 1 hr at 37^0 C
3. After incubation, the chambers were rinsed with PBS, the plastic case removed, and the cells were stained by fluorescent antibody technique.

Results and conclusion

Immunocytochemistry results showed that, in the presence of fresh mouse serum, more *T. congolense* had been engulfed by macrophages when the IgM anti-VSG concentration had been increased. Without IgM anti-VSG antibody, only a negligible

number of *T. congolense* were ingested. At antibody concentration 25 μg/ml, almost 100% of cells showed phagocytosis.

This result indicates that IgM anti-VSG-mediated phagocytosis of *T. congolense* by macrophages occurs in a dose-dependent pattern.

Figure 8.1

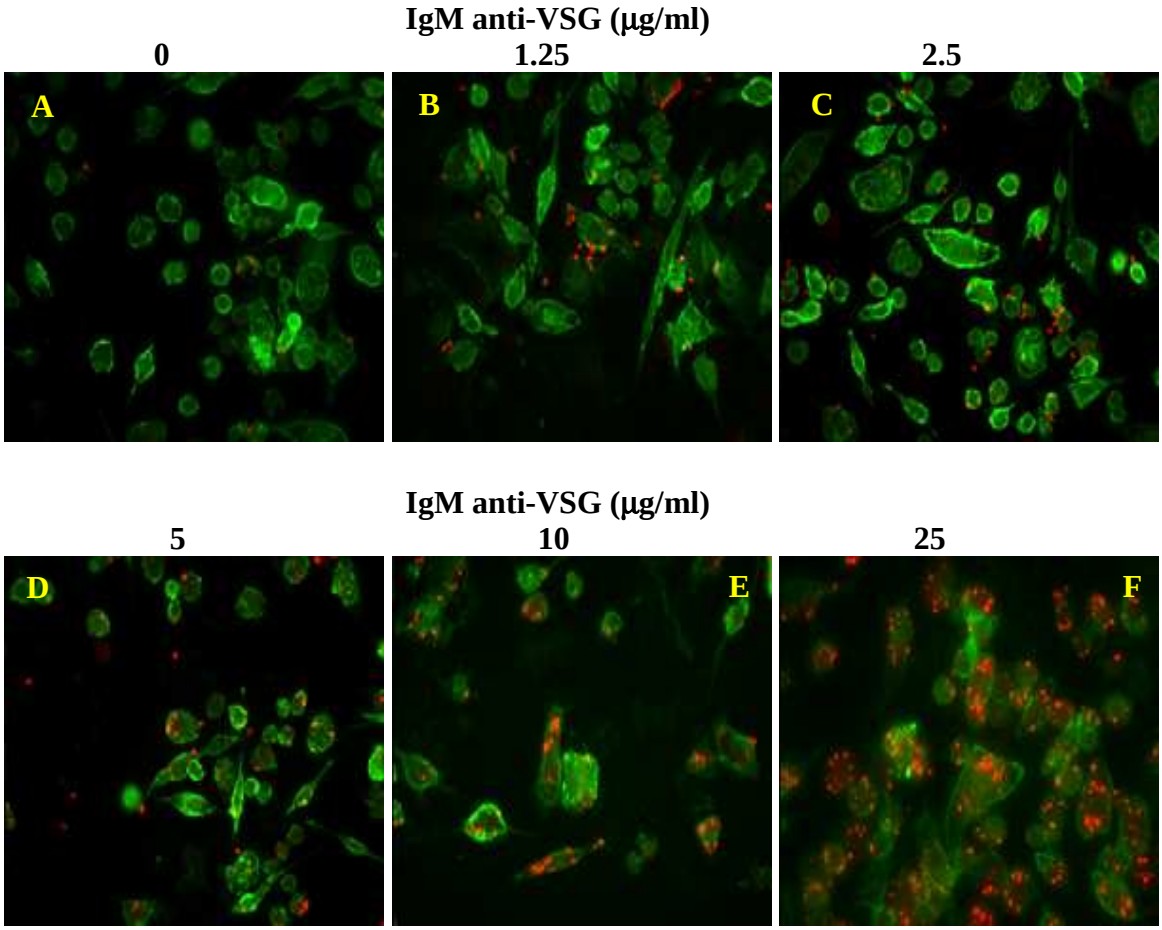


Figure 8.1 IgM anti-VSG mediates phagocytosis of *T. congolense* by macrophages cell line occurs in an antibody-dose-dependent pattern. Macrophages (BALB.BM cell line) were incubated with *T. congolense* for 60 min in the presence of 50% fresh CD1 mouse serum with different amounts of IgM anti-VSG (mAb 6C1. Phagocytosis of *T. congolense* was detected by immunocytochemical double staining. *T. congolense* was detected with the use of rabbit anti-*T. congolense* antiserum (red). Macrophages were detected with the use of anti-F4/80 (green) (400× magnification).

8.2 IgM anti-VSG-mediated phagocytosis of *Trypanosoma congolense* by macrophages increases with time of incubation (using BALB.BM cell line)

Aim

To test whether IgM anti-VSG-mediated phagocytosis of *T. congolense* by macrophages will increase with time of incubation

Protocol

1. Cells of macrophage cell line BALB.BM were seeded in Lab-Tek 16-chamber glass slides (Electron Microscopy Sciences, Washington, USA) at 5×10^4 cells per well in 200 μl DMEM complete medium with 12 ng/ml IFN-γ (R&D systems, Minneapolis, USA) for 24 hr at 37^0C.
2. The cells were washed 2x with DMEM. The mixture of freshly isolated *T. congolense* (macrophage: trypanosome ratio = 1:10) with IgM anti-VSG (mAb 6C1) (25 μg/ml) and 50% fresh CD1 mouse serum were added in the cell and cultured for different period of time at 37^0 C.
3. After incubation, the chambers were rinsed with PBS, the plastic case removed, and the cells were stained by fluorescent antibody technique.

Results and conclusion

Immunocytochemistry results showed that more IgM anti-VSG-mediated phagocytosis of *T. congolense* occurred with increasing time of incubation. The phagocytosis reached the maximum at an incubation time of 60 min. This result indicates that there is a positive correction between the degree of the IgM anti-VSG-mediated phagocytosis of *T. congolense* and time of incubation.

Figure 8.2

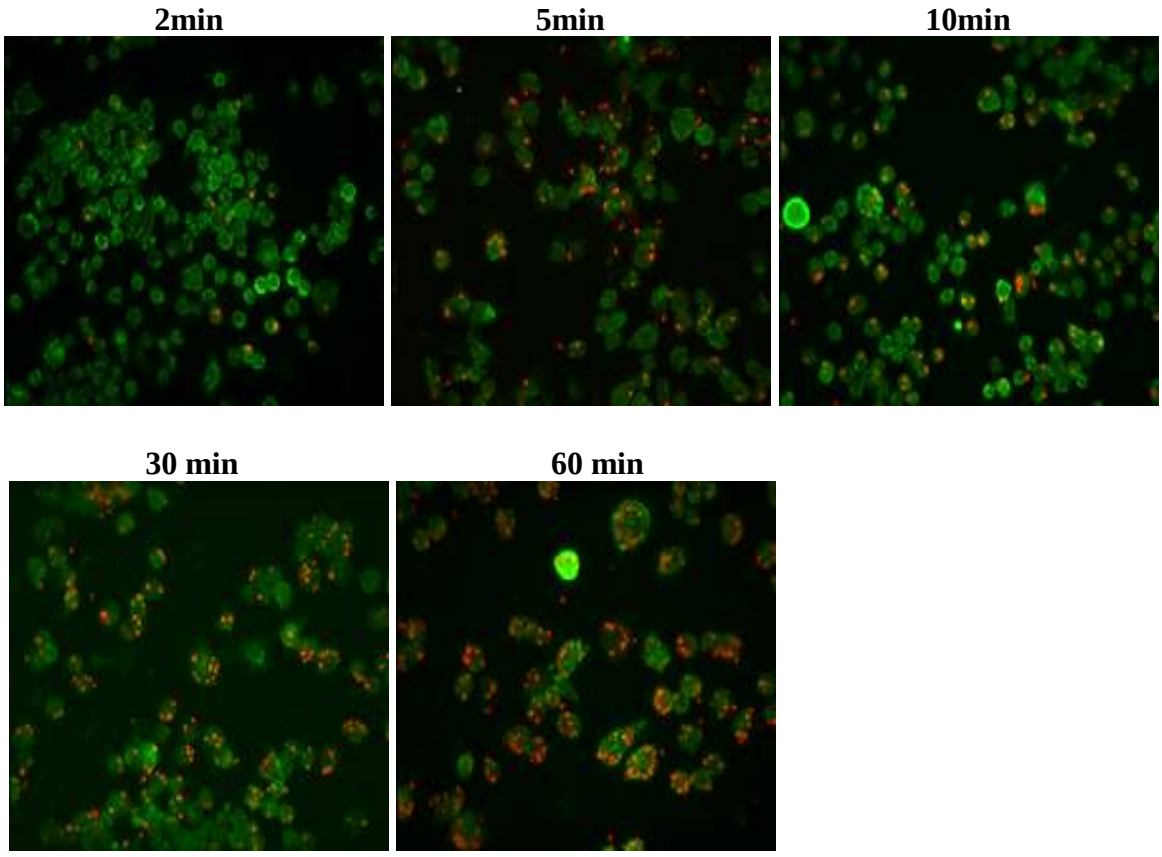


Figure 8.2 IgM anti-VSG-mediated phagocytosis of *T. congolense* by macrophages increases with time of incubation. Macrophages (BALB.BM cell line) were incubated with *T. congolense* and IgM anti-VSG (mAb 6C1) (25 μg/ml) in the presence of 50 % fresh CD1 mouse serum for different periods of time (2 min, 5 min, 10 min, 30 min and 60min). Phagocytosis of *T. congolense* was detected by immunocytochemical double staining. *T. congolense* was detected with the use of rabbit anti-*T. congolense* antiserum (red). Macrophages were detected with the use of anti-F4/80 (green) (400× magnification).

8.3 IgM anti-VSG-mediated phagocytosis of *Trypanosoma congolense* by CD11b-deficient or by normal peritoneal macrophages occurs in an antibody-dose-dependent pattern

Aim

To test whether the phagocytosis of *T. congolense* by macrophages from CD11b-deficient and normal mice will increase with increase of the concentration of IgM anti-VSG antibody

Protocol

1. Peritoneal macrophages were isolated from 8 to 10-week-old wild-type and CD11b-deficient C57BL/6 mice 3 days following i.p. injection of 0.3 ml pristane (Sigma, Oakville, Ontario, Canada). Peritoneal cells were collected, washed twice in DMEM complete medium, and resuspended in DMEM complete medium. Peritoneal cells were allowed to adhere to plastic tissue culture dishes for 2 h at 37^0C, after which non-adhered cells were removed by rinsing the cell monolayer with medium.
2. Peritoneal macrophages from CD11b-deficient and normal C57BL/6 mice were seeded in Lab-Tek 16-chamber glass slides (Electron Microscopy Sciences, Washington, USA) at 5×10^4 cells per well in 200 µl DMEM complete medium with 12 ng/ml IFN-γ (R&D systems, Minneapolis, USA) and incubated for 24 hr at 37^0C.
4. The cells were washed 2x with DMEM. The mixture of freshly isolated *T. congolense* (macrophage: trypanosome ratio = 1:10), 50% fresh CD1 mouse

serum and different amounts of IgM anti-VSG were added to the cells and cultured for 1 hr at 37^0 C.

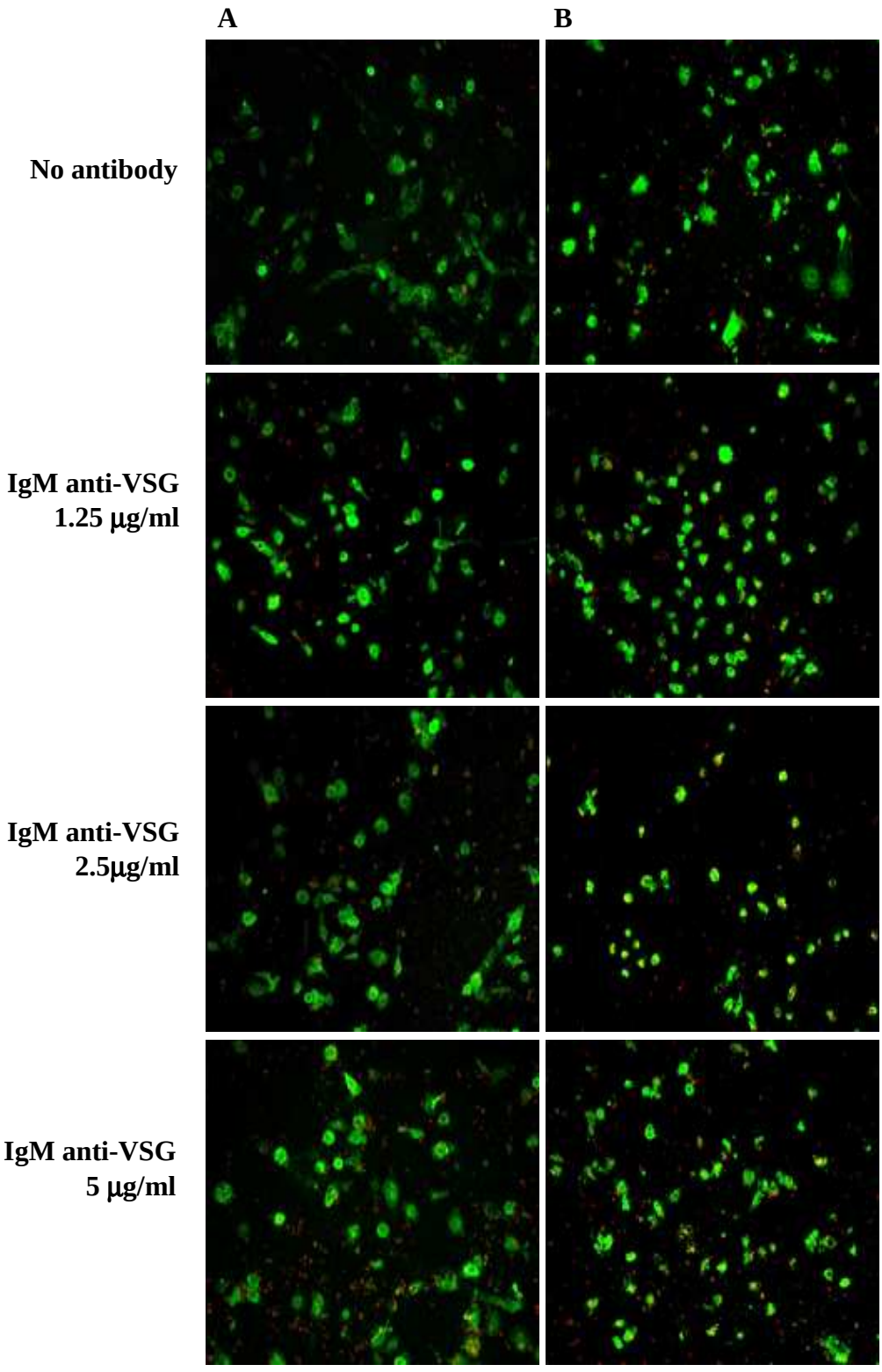
5. After incubation, the chambers were rinsed with PBS, the plastic case removed, and the cells on the microscope slide were stained with fluorescent antibodies.

Results and conclusion

Immunocytochemistry results showed that, in the presence of fresh mouse serum, the degree of IgM anti-VSG-mediated phagocytosis of *T. congolense* correlated with the increase of the antibody concentration. This happened with both normal and CD11b-deficient macrophages. Without IgM anti-VSG antibody, only a negligible number of *T. congolense* were ingested in both kinds of macrophages. At an antibody concentration of 25 μg/ml, almost 100% of cells from normal mice showed phagocytosis. There was a clear difference of phagocytosis between CD11b-deficient and normal macrophages at an antibody concentration of 10 μg/ml. The difference did not increase at higher antibody concentration.

These results indicate that IgM anti-VSG-mediated phagocytosis of *T. congolense* by macrophages from CD11b-deficient or normal mice occur in a dose-dependent pattern.

Figure 8.3


A
B
No antibody
IgM anti-VSG 1.25 µg/ml
IgM anti-VSG 2.5µg/ml
IgM anti-VSG 5 µg/ml

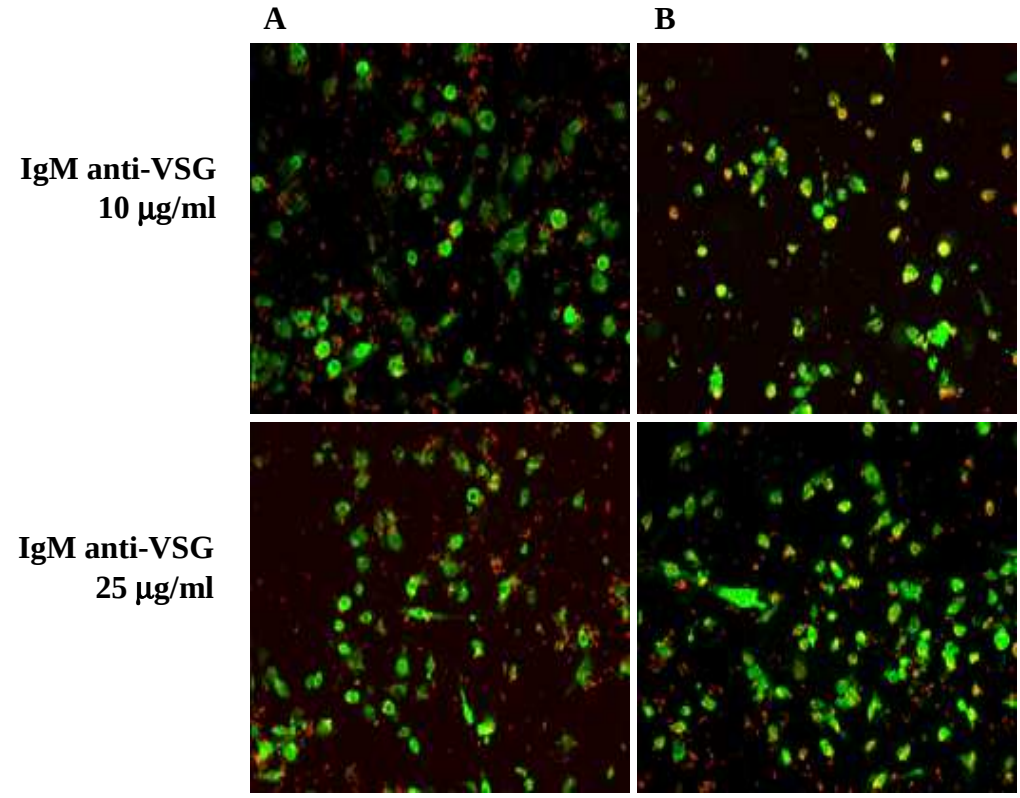


Figure 8.3 IgM anti-VSG-mediated phagocytosis of *T. congolense* by peritoneal macrophages from CD11b-deficient or normal C57BL/6 mice occur in an antibody-dose-dependent pattern. Macrophages from CD11b-deficient (Lane A) and normal C57BL/6 mice (Lane B) were incubated with *T. congolense* and different amounts of IgM anti-VSG (mAb 6C1) in the presence of 50% fresh CD1 mouse serum for 60 min. Phagocytosis of *T. congolense* was detected by immunocytochemical double staining. *T. congolense* was detected with the use of rabbit anti-*T. congolense* antiserum (red). Macrophages were detected with the use of anti-F4/80 (green) (200× magnification).

8.4 CR3 is the major receptor on macrophages involved in IgM anti-VSG-mediated phagocytosis of *Trypanosoma congolense*

Aim

To test whether CR3 is one of the receptors involved in IgM anti-VSG-mediated phagocytosis of *T. congolense*

Protocol

1. Peritoneal macrophages were isolated from 8 to 10-week-old wild-type and CD11b-deficient C57BL/6 mice 3 days following i.p. injection of 0.3 ml pristane (Sigma, Oakville, Ontario, Canada). Collected peritoneal cells were washed twice in DMEM complete medium, and resuspended in DMEM complete medium. Peritoneal cells were allowed to adhere to plastic tissue culture dishes for 2 h at 37^0C, after which non-adhered cells were removed by rinsing the cell monolayer with medium.
2. Peritoneal macrophage from CD11b-deficient and normal C57BL/6 mice were seeded in Lab-Tek 16-chamber glass slides (Electron Microscopy Sciences, Washington, USA) at 5×10^4 cell per well in 200 µl DMEM complete medium with 12 ng/ml IFN-γ (R&D systems, Minneapolis, USA) and incubated for 24 hr at 37^0C.
3. The cells were washed 2x with DMEM. The mixture of freshly isolated *T. congolense* (macrophage: trypanosome ratio = 1:10), IgM anti-VSG (mAb 6C1) (10 µg/ml) and 50% fresh CD1 mouse serum were added to the cells and cultured for 1 hr at 37^0 C.

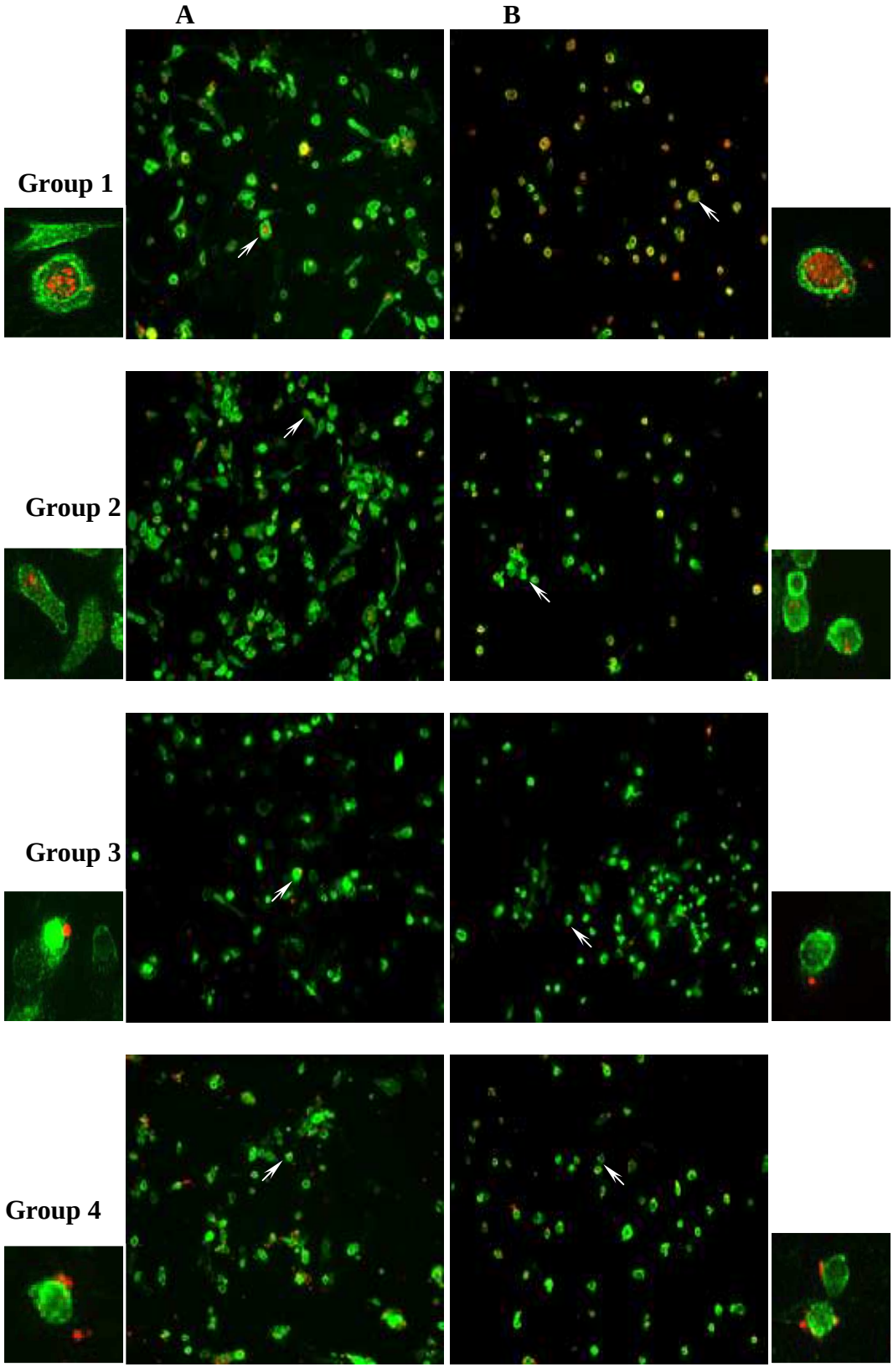
4. After incubation, the chambers were rinsed with PBS, the plastic case removed, and the cells on the microscope slide were stained by fluorescent antibody technique.

Results and conclusion

Immunocytochemistry results showed that, in the presence of fresh mouse serum, there occurred more IgM anti-VSG-mediated phagocytosis of *T. congolense* by normal macrophages than by CD11b-deficient macrophages. Without IgM anti-VSG antibody, only a negligible number of *T. congolense* were ingested in both kinds of macrophages. Without complement, a similar degree of phagocytosis was observed between normal and CD11b-deficient macrophages. The intracellular localization of *T. congolense* ingested by macrophages was confirmed by comparing the immunocytochemistry results i.e. when the procedure was preformed with (Group 1, 2, 3) or without (Group 4, 5) permeabilization of the cells by saponin.

These results indicate that CR3 (CD11b/CD18) is one of the receptors on macrophages involved in IgM anti-VSG-mediated phagocytosis of *T. congolense*, and the IgM anti-VSG-mediated phagocytosis of *T. congolense* via CR3 is entirely complement-dependent.

Figure 8.4



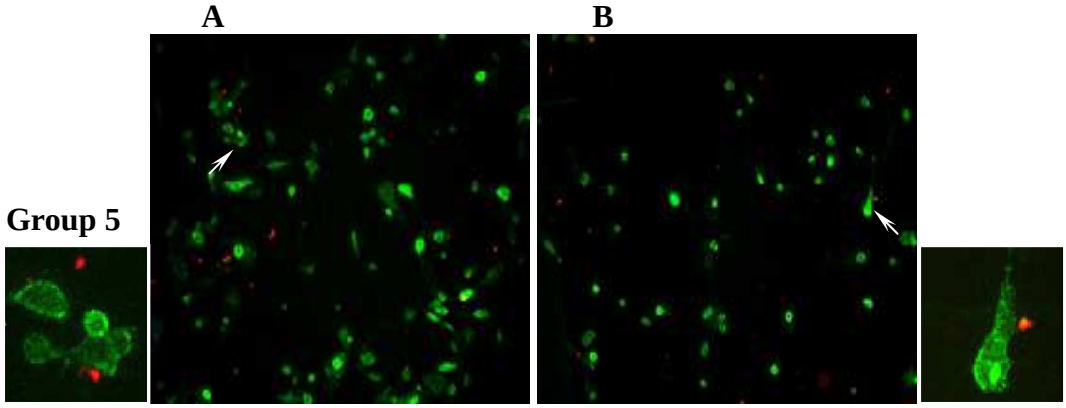


Figure 8.4 CR3 is the major receptor on macrophages involved in IgM anti-VSG-mediated phagocytosis of *T. congolense.* Macrophages from CD11b-deficient (Lane A) and normal C57BL/6 mice (Lane B) were incubated with *T. congolense* and IgM anti-VSG (mAb 6C) (10 μg/ml) in the presence or absence of 50% fresh CD1 mouse serum for 60 min. Phagocytosis of *T. congolense* was detected by immunocytochemical double staining. *T. congolense* was detected with the use of rabbit anti-*T. congolense* antiserum (red). Macrophages were detected with the use of anti-F4/80 (green) (200× magnification). The intracellular localization of *T. congolense* ingested by macrophages was confirmed by comparing the immunocytochemistry results with (Group 1, 2, 3) and without (Group 4, 5) permeabilization of cells by saponin.

Group 1: *T. congolense* + IgM anti-VSG + 50% fresh mouse serum (permeabilization of cells by saponin)

Group 2: *T. congolense* + IgM anti-VSG + 50% heat-inactivated mouse serum (permeabilization of cells by saponin)

Group 3: *T. congolense* + 50% fresh mouse serum (permeabilization of cells by saponin)

Group 4: *T .congolense* + IgM anti-VSG + 50% fresh mouse serum (no permeabilization of cells by saponin)

Group 5: *T. congolense* + IgM anti-VSG + 50% heat-inactivated mouse serum (no permeabilization of cells by saponin)

8.5 Release of sVSG from *Trypanosoma congolense* by interaction with anti-VSG antibody correlates with the time of incubation

Aim

To test whether antibody-induced release of sVSG from *T. congolense* is affected by the incubation time

Protocol

1. 10^8 /ml *T. congolense* were mixed with anti-VSG antibody (the concentration of IgM mAb 6C1, IgG2a mAb1D11 or mouse IgM was 25 μg/ml) and 50% fresh CD1 mouse serum in TSG. The mixtures were incubated at 37^0C for different periods of time, and then centrifuged at 13,000 x g at 4^0C for 15 min. The supernatants were collected.
2. The sVSG in the supernatants were tested by western blot. Rabbit anti-*T. congolense* antiserum was used as first antibody, and peroxidase-labelled mouse anti-rabbit IgG (H+L) as second antibody.

Results and conclusion

Western blot results showed that the degree of release of sVSG from *T. congolense* correlated with the time of incubation. These results indicate that optimal anti-VSG-induced release of sVSG from *T. congolense* requires about 60 min.

Figure 8.5

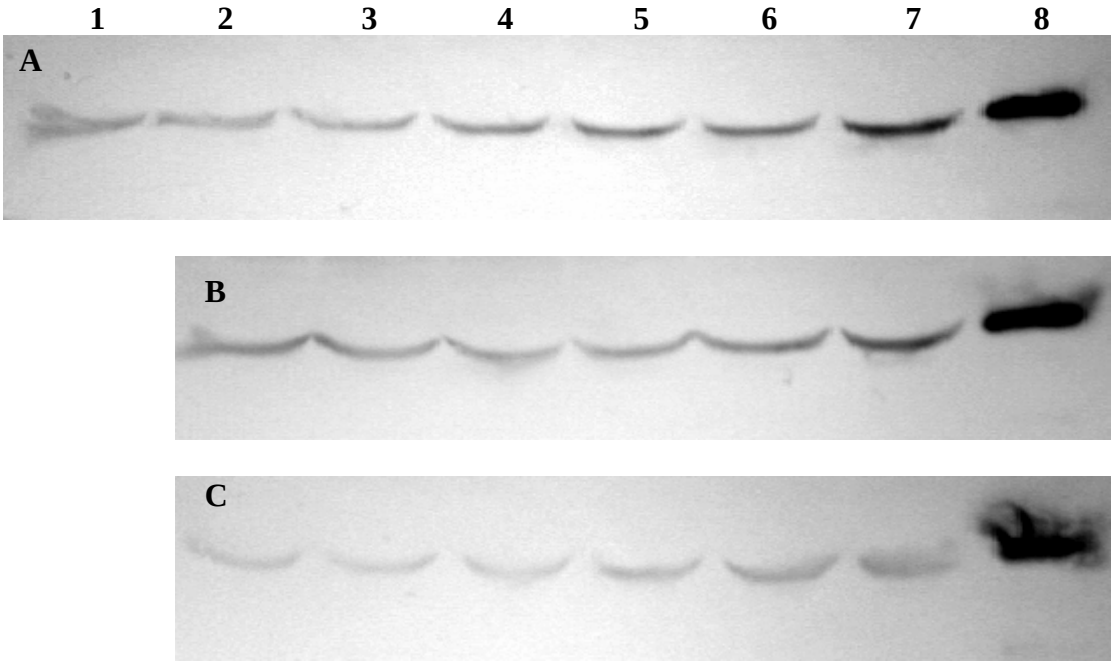


Figure 8.5 The degree of anti-VSG antibody-induced shedding of sVSG from *T. congolense* correlates with increase of incubation time. In the presence of 50% fresh CD1 mouse serum, *T. congolense* (10^8/ml) were mixed with IgM anti-VSG (mAb 6C1) (A), IgG anti-VSG (mAb 1D11)(B) or mouse IgM (isotype control)(C). The mixtures were incubated at 37^0C for different period of time, and, for separating the supernatant, they were spun down for 15 min at 13,000 x g at 4^0C. The sVSG in the supernatants were detected by Western blot ($1^{\#}$ Ab: rabbit anti-*T. congolense* antiserum, $2^{\#}$ Ab: peroxidase-labelled mouse anti-rabbit IgG (H+L)).

Lane 1: incubation time 0min

Lane 2: incubation time 2 min

Lane 3: incubation time 5 min

Lane 4: incubation time 10 min

Lane 5: incubation time 20 min

Lane 6: incubation time 30 min

Lane 7: incubation time 60 min

Lane 8: partly purified sVSG

8.6 Fresh serum from C57BL/6 mice enhances IgG2a anti-VSG-induced release of sVSG from *T. congolense* more than fresh serum from BALB/c mice

Aim

To test the effect of fresh C57BL/6 serum and BALB/c serum on the release of sVSG from *T. congolense* by interaction with IgG2a anti-VSG

Protocol

1. 10^7 /ml *T. congolense* were mixed with IgG2a anti-VSG (mAb 1D11) (5 μg/ml or 2.5 μg/ml) and 50% fresh CD1 mouse serum in TSG. The mixtures were incubated at 37^0C different for 30 min, and then centrifuged at 13,000 x g at 4^0C for 15 min. The supernatants were collected.
2. The sVSG in the supernatants were tested by western blot. Rabbit anti-*T. congolense* antiserum was used as first antibody, and peroxidase-labelled mouse anti-rabbit IgG (H+L) as second antibody.

Results and conclusion

Western blot results showed that more antibody-induced release of sVSG from *T. congolense* occurs in the presence of fresh C57BL/6 serum than in the presence of fresh BALB/c serum. These results indicate IgG2a anti-VSG-induced release of sVSG from *T. congolense* is affected by the source of complement.

Figure 8.6

IgG2a anti-VSG (μg/ml)	5	5	2.5	2.5	0	0
C57BL/6 serum	+	-	+	-	+	-
BALB/c serum	-	+	-	+	-	+
Dand density	**240**	**118**				

Figure 8.6 Fresh serum from C57BL/6 mice enhances IgG anti-VSG-induced release of sVSG from *T. congolense* more than fresh serum from BALB/c mice. *T. congolense* (10^7/ml) were incubated with different amount of IgG anti-VSG (mAb 1D11) and 50% fresh C57BL/6 serum or fresh BALB/c serum. The supernatants of the mixtures were tested for sVSG by Western blot ($1^{\#}$ Ab: rabbit anti-*T. congolense* antiserum, $2^{\#}$ Ab: peroxidase-labelled mouse anti-rabbit IgG (H+L)). The band densities were measured by Alphaimager software.

8.7 Interaction of IgM anti-VSG and *Trypanosoma congolense* in the presence of complement: some of the shed sVSG is covalently bound to C3 fragments (sVSG-C3b)

Aim

To test whether sVSG being released from *T. congolense* covalently binds with C3 fragment

Protocol

1. 10^8 /ml *T. congolense* were mixed with IgM anti-VSG antibody (25 μg/ml) and 50% fresh mouse serum with or without 10mM EDTA. Fresh C57BL/6 serum or fresh BALB/c serum were used. The mixtures were incubated at 37^0C for 30 min, and then centrifuged at 13,000 x g at 4^0C for 15 min. The supernatants were collected. EDTA is chelating Ca^{++} and Mg^{++} and used as inhibitor of the complement cascade
2. All supernatants were mixed with rabbit anti-*T. congolense* antiserum in the presence of 10 mM EDTA at RT for 1 hr. They were then centrifuged at 13,000 x g at 4^0C for 15 min. The precipitates were washed with PBS and then suspended in PBS/1% SDS. The precipitates were tested for C3 fragments by western blot (peroxidase-labelled goat anti-mouse C3), as well as for sVSG by western blot ($1^{\#}$: rabbit anti-*T. congolense* antiserum; $2^{\#}$: peroxidase-labelled mouse anti-rabbit IgG (H+L)

Results and conclusion

A band of molecular weight of about 210kD, corresponding to the size of C3b-VSG, was detected in the presence of fresh mouse serum without EDTA (A, lane 2 & 4) but

not in fresh mouse serum with 10mM EDTA (A, lane 3 & 5). A band of similar size was detected by rabbit anti-*T. congolense* antiserum in the presence of fresh mouse serum without use of EDTA (B, lane 2 & 4), but not in the presence of fresh mouse serum with 10 mM EDTA (B, lane 3 & 5). These results indicate that some sVSG shed from *T. congolense* covalently binds with a C3 fragment.

Figure 8.7

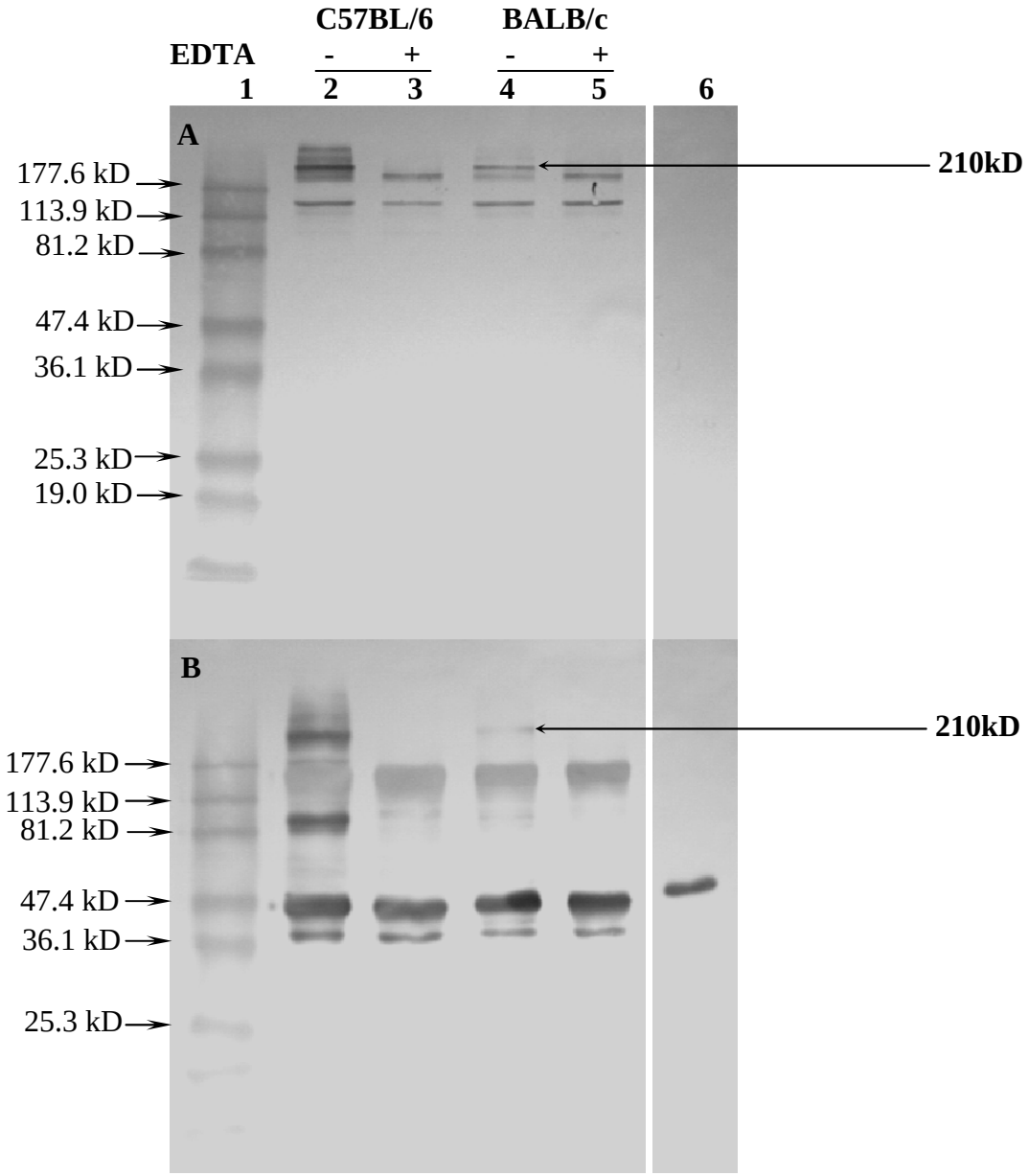


Figure 8.7 Interaction of IgM anti-VSG and *Trypanosoma congolense* in the presence of complement: some of the shed sVSG is covalently bound to C3 fragments (sVSG-C3b). Immunoprecipitations by anti-*T. congolense* antiserums were carried out using the supernatants from the incubated mixtures of *T. congolense* (10^8/ml), IgM anti-VSG (mAb 6C1) (25 μg/ml) and 50% of fresh mouse serum with or without 10 mM EDTA. Fresh mouse sera from C57BL/6 or BALB/c mice were used. The precipitates from the immunprecipitation were tested for C3 fragments by western blot (peroxidase-labelled goat anti-mouse C3 IgG [Cappel]) (A), and tested for sVSG by western blot ($1^{\#}$ Ab: rabbit anti-*T. congolense* antiserum, $2^{\#}$ Ab: peroxidase-labelled mouse anti-rabbit IgG (H+L)) (B).

Lane 1: BenchMark prestained protein ladder;

Lane 2: fresh C57BL/6 serum+ *T. congolense* + IgM anti-VSG;

Lane 3: fresh C57BL/6 serum + 10mM EDTA+ *T. congolense*+ IgM anti-VSG;

Lane 4: fresh BALB/c serum + *T. congolense*+ IgM anti-VSG;

Lane 5: fresh BALA/c serum + 10mM EDTA+ *T. congolense*+ IgM anti-VSG;

Lane 6: partly purified sVSG.

8.8 Parasitemia in CD11b-deficient and normal C57BL/6 mice infected with *Trypanosoma congolense*

Aim

To test the role of CR3-mediated phagocytosis of *T. congolense* in controlling parasitemia in vivo

Protocol

1. 5 female 10 to 12 week-old CD11b-deficient mice and 4 female 10 to 12 week-old normal C57BL/6 mice were injected with 10^3 *T. congolense*.
2. The parasitemia was estimated by examining a drop of blood from the tail vein on a microscopic slide at 400x magnification by phase contrast microscopy.

Result and conclusion

CD11b-deficient mice control the first parasitemia earlier than normal C57BL/6 mice. At day 7-post infection, CD11b-deficient mice showed significant lower parasitemia than normal mice. These data suggest that CR3-deficience might weakly enhance, rather than decrease, the control of parasitemia in *T. congolense* infection.

Figure 8.8

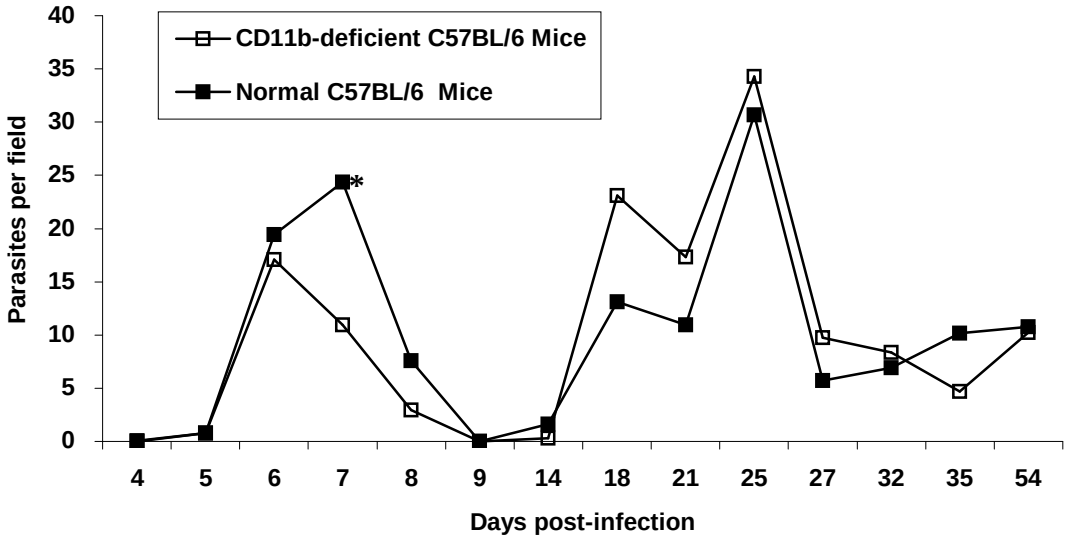


Figure 8.8 Parasitemia in CD11b-deficient mice and normal mice infected with *Trypanosoma congolense.* 5 female 10 to 12 week-old CD11b-deficient mice and 4 female 10 to 12 week-old normal C57BL/6 mice were injected with 10^3 *T. congolense* (ip.). The parasitemia was estimated by examining a drop of blood from the tail vein on a microscopic slide at 400× power by phase contrast microscopy. * On day 7, the parasitemia in CD11b-deficient mice was significantly lower than in normal C57BL/6 mice (10.92±2.82 vs. 24.35±0.35, respectively) (p=0.005).